Annual Report

Institute for Research in Biomedicine

2005 | 2006

Scientific Report

IRB
BARCELONA

INSTITUTE
FOR RESEARCH
IN BIOMEDICINE

Table of Contents

Foreword

Building an institute in Europe's newest city of science

Catalonia has produced many excellent scientists, but whereas it has held onto its artists and musicians, it has kept too few of its scientists. That is now changing quickly thanks to a dedicated effort on the part of the Catalan Government. Barcelona is becoming an international centre for science, and IRB Barcelona is a central figure in this process. Our vision is to make a vital contribution to the biomedical revolution that is now sweeping the world, one which promises to eradicate major diseases that have been beyond the reach of current medicine.

IRB Barcelona was founded by the Ministry of Innovation, Universities and Business and the Ministry of Health of the Government of Catalonia, the University of Barcelona and the Barcelona Science Park a mere two years ago, but we already comprise more than 350 research, technical and administrative staff. We will continue to grow over the next two or three years, adding new groups, platforms, and expertise that will widen our focus and bring in new disciplines. Yet even at the current size we are getting things done. This can be seen from the publications of our scientists, listed in this report, which have been achieved in the short time since the groups moved in.

Our vision is to make a vital contribution to the biomedical revolution that is now sweeping the world

The diseases that are now the major causes of death in the developed world arise from flaws in our own cells. Finding cures will require a deep understanding of their causes and input from genetics, cell and developmental biology, chemistry, physics, computation, and many other fields. Today these fields are coming together rapidly to approach medical questions in new ways. IRB Barcelona gathers them under one roof with a common aim: to learn to translate findings from basic research into medical applications.

Science at the IRB Barcelona is organised into five highly collaborative programmes, each with a core focus:

Structural and Computational Biology focuses on the structure of single molecules and their interactions, using methods from physics and computational science: X-rays, NMR, electron microscopy, macromolecular biophysics, bioinformatics and molecular modelling.

Chemistry and Molecular Pharmacology programme specialises in the design and synthesis of small molecules and macromolecules that can be used to proteins and other biological molecules.

Cell and Developmental Biology studies how information in the genome is used to create structures within the cell, to guide the formation and regeneration of tissues, and to create a whole organism. High-throughput methods are used to watch the global activity of genes and proteins in healthy and diseased organisms.

Molecular Medicine probes the molecular bases of metabolic and genetic diseases, searches for diagnostic or therapeutic targets, and studies the behaviour of the entire genome and proteome during diseases.

Oncology studies diverse aspects of how tumours arise and develop, the relationship between stem cells and cancer, and the identification of cellular programmes that cause particular types of tumours to spread and metastasise in specific parts of the body. This research line is directed by Joan Massagué and is operated in coordination with the Cancer Biology and Genetics programme that he chairs at the Memorial Sloan-Kettering Center, in New York.

IRB Barcelona gathers different fields under one roof with a common aim: to learn to translate findings from basic research into medical applications

We are committed to doing our work at an extremely high level and have put into place a management and advisory structure to help us achieve this. Our scientific work will be regularly assessed by an External Advisory Board which comprises 15 leading international researchers in biomedicine. Its main task is to provide guidance in shaping our research and related activities.

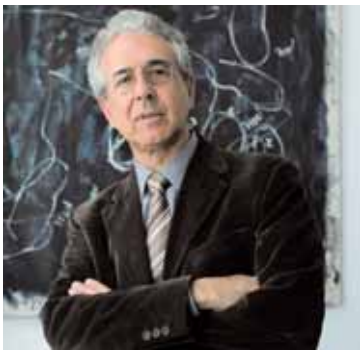
Research is most likely to achieve excellence with superb technical support and a fertile environment. We are in the process of setting up new service units to provide our researchers with state-of-the-art facilities. IRB Barcelona is ideally situated in the Barcelona Science Park complex,

where it can draw on an already considerable palette of service units established and operated by the Barcelona Science Park and by the Scientific and Technical Services of the University of Barcelona.

Training is one of the core missions of IRB Barcelona, and its largest plank is a dedicated PhD programme. Students at IRB Barcelona have an excellent opportunity to meet leading researchers in their respective fields through the Barcelona BioMed Seminar Series. We are also keeping abreast of developments in the field through a series of meetings called the Barcelona BioMed Conferences, organised in collaboration with the BBVA Foundation. They provide a new, creative platform where leading researchers can meet and discuss the most recent breakthroughs in several fields of the biomedical sciences. A constant flow of international visitors provides another crucial link to the world, both for the expertise they bring and the rich cultural experiences that arise from their stay. IRB Barcelona hosts visitors at all stages of their scientific careers.

Institutes need to discuss their research with the public and help prepare people for those changes. IRB Barcelona is actively explaining our work through the mass media at a level that people can understand, through education activities, and through the Barcelona BioMed Forums. These events are aimed at increasing public awareness and encouraging a better understanding of progress in the biomedical sciences.

Our aims are ambitious, and success will depend on both internal and external factors. We are building an institute for the future, and we are doing so at a very special time in science. Adapting to this changing environment will continue to require creativity and administrative flexibility. We have been able to draw on excellent support from the Board of Trustees and a high level of motivation from the staff. I thank all of you for your efforts in making the launch of this institute a great success.



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Chromatin structure and function

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The contribution of chromatin to the regulation of genomic functions is well established. Our knowledge about the regulation of chromatin functions has benefitted from the identification of components, and mechanisms, that modify the structural and functional properties of chromatin: chromatin assembly and remodeling complexes; histone modifications (*ie*, acetylation, methylation, phosphorylation, ubiquitination) and their corresponding enzymes; structural non-histone proteins (*ie*, HP1; Polycomb, PC) that recognise specific histone modifications and contribute to the structural properties of distinct chromatin domains; histone variants which localise to specific chromosomal locations (*ie*, CENP-A, H3.3, H2A.Z, macroH2A), *etc*. Our research focuses on the study of the molecular basis of chromatin function and its regulation.

Changes in chromatin structure are at the basis of many regulatory processes and, in particular, gene silencing frequently occurs at the chromatin level, being associated with the acquisition of a specific structural organisation (silent chromatin). Silent chromatin includes distinct chromatin domains: heterochromatin, both constitutive (*ie*, centromeric and telomeric) and facultative (*ie*, inactive X of mammals), as well as specific euchromatic loci that become silenced during cell differentiation and development (*ie*, mating type locus in yeasts, homeotic genes). In recent years, the basic mechanisms involved in the formation of silent chromatin have begun to be unraveled. Centromeric silencing, mediated by HP1, and silencing of the homeotic genes, which depends on PC-proteins, are among the best-characterised examples. In both cases, silencing involves the action of a histone methyltransferase enzyme (HMT) (*Su(var)3-9* for HP1 and *Enhancer of zeste* (*E(Z)*) for PC), which introduces a specific histone modification (H3K9me$_{2,3}$ for *Su(var)3-9* and H3K27me$_{2,3}$ for *E(Z)*) that, in turn, is recognised by the chromodomains of HP1 and PC, respectively. These generic interactions are crucial for the formation and maintenance of silent chromatin. In addition, the RNAi pathway appears to play an essential role in the initial steps of establishment of silencing. Our research focuses on the factors and mechanisms involved in the formation and maintenance of silent chromatin, and their functional contribution to: (i) the regulation of centromeric and telomeric function, and (ii) the regulation of homeotic gene expression.

Centromere identity and function
Rather than being associated with specific DNA sequences, centromeric function is determined by a specialised structural organisation, the centromeric chromatin, which results from the cooperation of two specific chromatin structures: (i) *centric chromatin*, characterised by the presence of a centric specific histone H3 variant (CENP-A), and (ii) *centromeric heterochromatin*, characterised by a specific pattern of histone modifications (H3K9me$_{2,3}$), recognised by heterochromatin associated proteins (HP1). (See Figure 1.)

CENP-A deposition determines centromere identity
Centromere identity is determined by the deposition of the centromere-specific histone H3 variant, CENP-A, which replaces canonical H3.1 in all eukaryotic centromeres. CENP-A is found exclusively at centromeres, recruits kinetochore components and is required for centromere function. How is centromere-specific deposition of CENP-A achieved? We are currently addressing this question in *Drosophila* (Moreno-Moreno *et al*, 2006). Contrary to canonical nucleosomes, deposition of CENP-A-containing nucleosomes at centromeres is not linked to DNA replication. Deposition of CENP-A nucleosomes is, however, a promiscuous process as it can also occur during DNA

replication leading to its mislocalisation throughout chromatin. Expression of CENP-A must, therefore, be tightly regulated during cell cycle progression to prevent replication-dependent deposition at non-centromeric sites during S-phase. In fact, mammalian CENP-A is only expressed during G2-phase. However, expression of the *Drosophila* homologue of CENP-A (CID) takes place early during S-phase. Therefore, additional mechanisms must exist to either avoid deposition of CENP-A containing nucleosomes at non-centromeric sites during DNA replication and/or to remove them afterwards. Our work shows that proteasome-mediated degradation restricts localisation of CID to centromeres by eliminating mislocalised CID as well as by regulating available CID levels. Regulating available CID levels is essential to ensure centromeric deposition of CID. Proteasome mutants show increased expression and mislocalisation of CID. (See Figure 2.) Consistent with a role on kinetochore assembly, mislocalisation of CID-nucleosomes induces cell cycle arrest. Actually, binding of CID at ectopic sites recruits kinetochore components, an effect depends on the N-terminal domain of CID. Proteasome-mediated degradation appears to be an evolutionarily conserved mechanism that regulates available CENP-A levels to favor its preferential deposition at centromeres as, also in the yeast *Saccharomyces cerevisiae*, the levels of CENP-A (Cse4p) are regulated by the proteasome and proteolysis-resistant mutants mislocalise throughout chromatin. Proteasome-mediated degradation of CENP-A was also reported in human cells infected with herpes simplex virus type 1 (HSV-1). How is CENP-A proteolysis regulated? In this respect, we have identified in a yeast two-hybrids screen the interaction of CID with partner of paired (Ppa), an F-box containing protein that interacts with Skp1, an evolutionarily conserved component of the SCF ubiquitin ligase complex.

Heterochromatin structure and function: the contribution of vigilin

Vigilin is a highly evolutionarily conserved protein, from yeasts to humans, which is characterised by the presence of multiple KH-domains. The KH-domain is a single-stranded nucleic-acids binding motif that, first identified in the RNA binding protein hnRNPK, has been found in a number of proteins binding single-stranded nucleic-acids. Vigilin participates in different aspects of RNA metabolism, and binds soluble and membrane-bound polyribosomes. In addition, vigilin contributes to heterochromatin structure and function. In *Drosophila*, vigilin (DDP1), contributes to the structure and function of centromeric heterochromatin (Huertas *et al*, 2004). Centromeric silencing, H3K9 methylation and HP1 deposition are affected in DDP1 mutants. Furthermore, DDP1 mutants show defects on chromosome condensation and segregation. Also *S. cerevisiae*, vigilin (Scp160p) is involved in the control of cell ploidy and contributes to heterochromatin-induced silencing (Cortés *et al*, 1999; Marsellach *et al*, 2006). A îscp160 deletion relieves silencing both at telomeres and at the mating-type locus, but not ribosomal silencing. Loss of telomeric silencing is associated to a decreased Sir3p deposition but is independent of binding of Scp160p to ribosomes. What is the mechanism underlying the contribution of vigilin to heterochromatin structure and function? Heterochromatin clusters at the nuclear envelope and binding to the nuclear envelope helps to establish heterochromatin-induced silencing. Vigilin appears to play a role in the interaction of het-

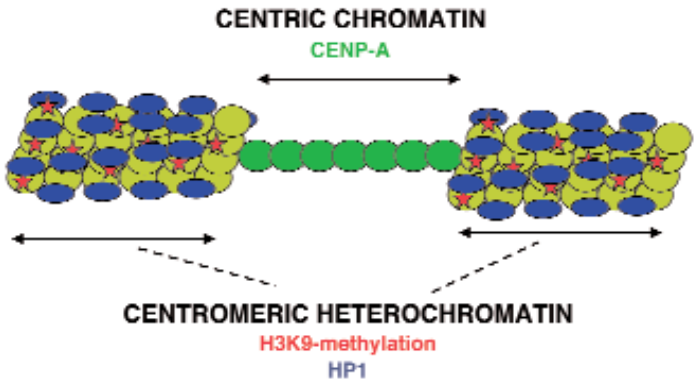

Figure 1. Centromeric function is determined by a specialised chromatin organisation, which results from the cooperation of two specific chromatin structures: centric chromatin, which carries the centromeric specific histone H3 variant (CENP-A), and centromeric heterochromatin that is characterised by binding of HP1, which recognises a specific pattern of histone methylation H3K9me2,3.

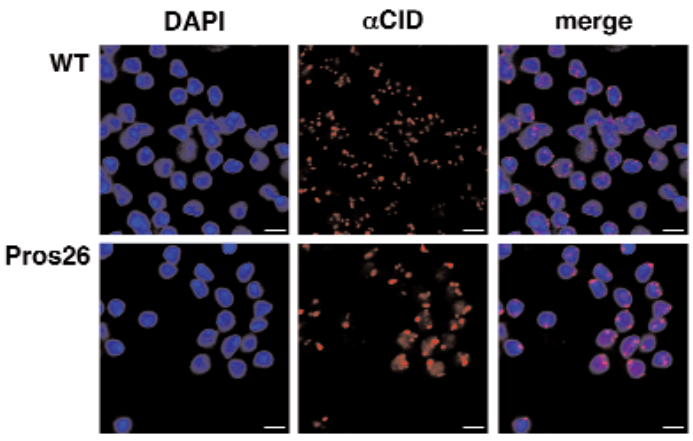

Figure 2. Proteasome-mediated degradation restricts localisation of CID, the centromeric specific histone H3 variant of Drosophila*, to centromeres. In neuroblast cells from wild type flies (WT), CID localises exclusively at centromeres but mutations in proteasome subunits (Pros26) result in increased expression and delocalisation of CID.*

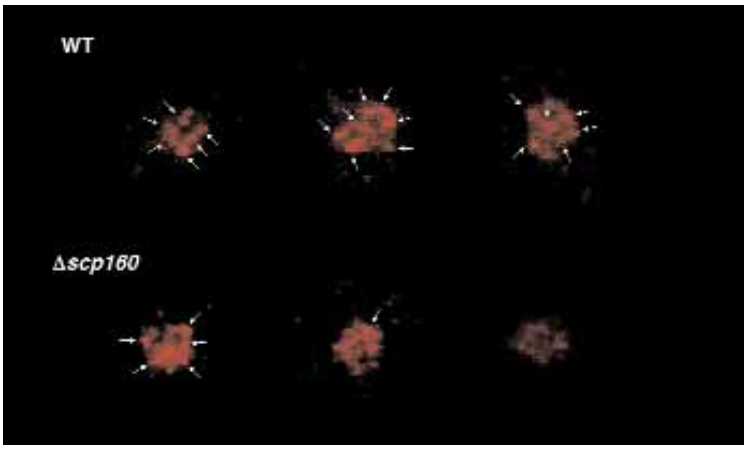

Figure 3. In S. cerevisiae, *vigilin (Scp160) contributes to clustering of heterochromatin at the nuclear envelope. Several heterochromatin clusters at the nuclear envelope (arrows) are detected in wild type cells (WT) but not in îscp160 cells.*

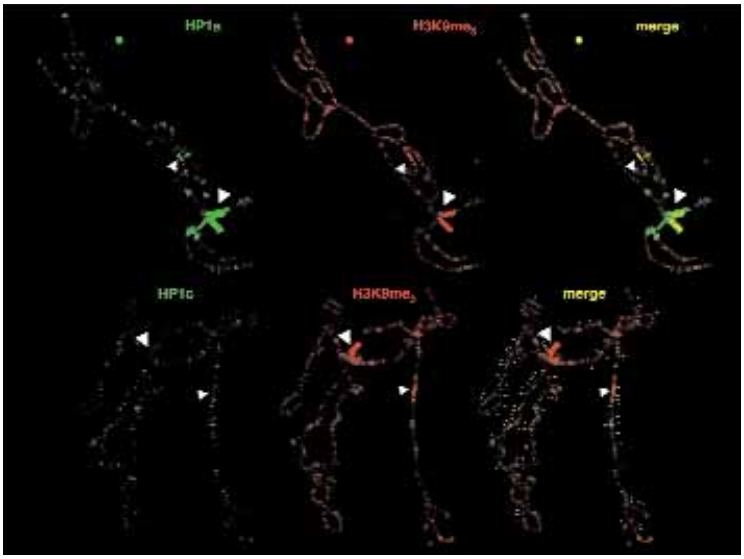

Figure 4. HP1 isoforms show differential patterns of chromosomal distribution. In Drosophila *polytene chromosomes, HP1a localises at the heterochromatic chromocentre, co-localising with H3K9me2,3, and at a few euchromatic sites, where it does not co-localise with H3K9me2,3. On the other hand, HP1c localises exclusively to euchromatin where it shows a significant co-localisation with H3K9me2,3.*

erochromatin with the nuclear envelope. In yeasts (*S. cerevisiae* and *S. pombe*), *Drosophila* and vertebrates (chicken and humans) vigilin shows a perinuclear localisation and, in human cells, vigilin interacts with Ku70/86, an heterochromatin-associated factor involved in anchoring heterochromatin to the nuclear envelope. Moreover, in *S. cerevisiae*, clustering of telomeric heterochromatin at the nuclear envelope is perturbed in îscp160 cells (see Figure 3).

HP1 isoforms

HP1 plays and essential role in the formation and maintenance of centromeric heterochromatin. Three HP1 isoforms exist in *Drosophila* (HP1a, HP1b and HP1c), which share highly conserved chromo- and chromo-shadow domains. The chromodomain is involved in specific recognition of H3K9me2,3 and the chromo-shadow domain mediates protein-protein interactions. Despite their similarities, the three HP1 isoforms show different patterns of localisation (Font *et al*, in preparation). In polytene chromosomes, HP1a is mostly located at centromeric heterochromatin. This localisation depends on Su(var)3-9 and, therefore, on H3K9 methylation. HP1a also localises to some euchromatic regions but, at these locations, it does not always co-localise with H3K9me2,3, nor it induces silencing. In addition, euchromatic deposition of HP1a does not depend on Su(var)3-9. On the other hand, HP1c shows a strictly euchromatic localisation that does not depend either on Su(var)3-9 but, though partially, co-localises with H3K9me3. Finally, HP1b shows a mixed localisation pattern being present both at centromeric heterochromatin and at distinct euchromatic sites (See Figure 4). Also in mammals, there are three HP1 isoforms which show a similar behaviour. These observations indicate that chromosomal deposition of HP1 is not exclusively determined by the state and type of histone methylation. Interactions with elements involved in chromatin assembly, DNA replication and transcription are likely to play an essential role in bringing HP1 isoforms to chromatin. What is, then, the role played by histone methylation? HP1 isoforms can interact with unmethylated histones, as well as with DNA and RNA, but they preferentially bind nucleosomes containing H3K9me2,3 (Font *et al*, in preparation), indicating that recognition of H3K9me2,3 stabilises binding of HP1 to chromatin. From these observations, a model emerges where HP1 is brought to chromatin by factors involved in various chromatin transactions and, then, lock-in at these sites by histone methylation and H3K9me2,3 recognition. Actually, complexes involved in HP1 deposition might themselves carry HMT activities.

Telomere structure and function

Telomeres are complex nucleo-protein structures that play important roles in tumourogenesis, senescence, genome instability and cell cycle progression. Telomeres protect chromosomes from the terminal erosion that takes place every cell division and, in addition, they constitute a reservoir of proteins known to participate in multiple cellular processes such as DNA repair and recombination. *Drosophila* telomeres are of an unusual class. In most eukaryotes, telomeres are composed of short repetitive guanine rich sequences and they are replicated by an

specialised ribo-nucleoprotein enzyme, the telomerase. On the other hand, though functionally equivalent to classical telomeres, *Drosophila* telomeres are made of successive transpositions of three retrotransposons, HeT-A, TART and TAHRE, which transpose exclusively to the end of chromosomes (See Figure 5.) This is specially interesting because retrotransposons have been classically considered deleterious, or at least parasitic, genomic elements. *Drosophila* telomeres constitute a good example of how retrotransposons have changed their parasitic nature into an indispensable cellular role.

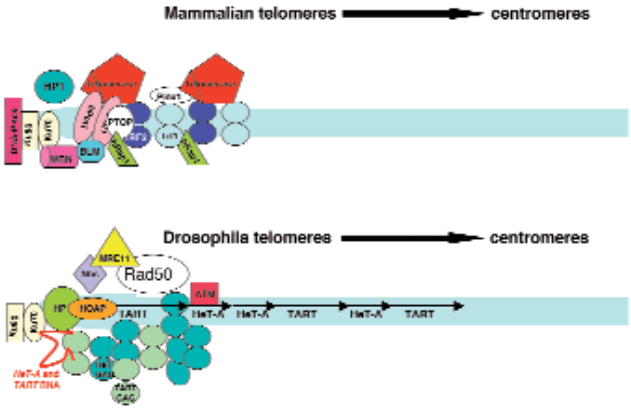

Figure 5. Structural organisation of mammalian and Drosophila *telomeres.*

Telomere targeting

Which are the mechanisms that target the telomeric retrotransposons exclusively to telomeres? The Gag protein of HeT-A plays a central role. Because of their essential role, it was assumed that telomeric retrotransposons would be highly evolutionary conserved among all *Drosophila* species. Unexpectedly, we found that, while maintaining their unique genomic organisation, telomeric retrotransposons show significant divergence with the exception of a few highly conserved motifs (Casacuberta and Pardue, 2003a and 2003b). However, in species as distant as *D. melanogaster* and *D. virilis*, the HeT-A element conserves most of its unusual characteristics while maintaining only a 21 % of amino acid identity. Most important, both in *D. virilis* and *D. melanogaster*, HeT-A Gag localises to telomeres in interphase cells and, in *D. melanogaster*, it is required for telomere targeting of the other telomeric retrotransposons. Moreover, the HeT-A Gag protein of *D. virilis* also localises to telomeres when expressed in *D. melanogaster* cells and, vice versa, the HeT-A Gag protein of *D. melanogaster* localises to telomeres in *D. virilis* (Casacuberta *et al*, 2007. (See Figure 6.) These observations indicate that both the protein and the cellular determinants to correctly drive HeT-A Gag to telomeres have been conserved throughout 60 MY of evolution.

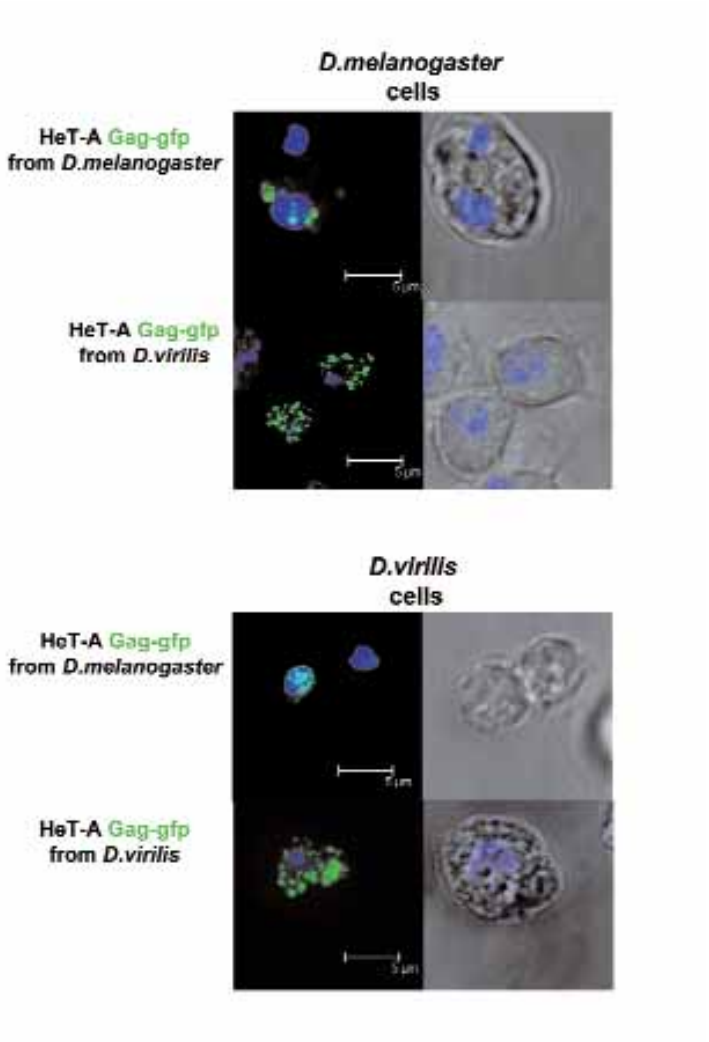

Figure 6. The Gag protein of the telomeric retrotransposon HeT-A localises to telomeres both in distant Drosophila *species such as* D. melanogaster *and* D. virilis.

Which other cellular components might be important for telomere targeting of telomeric retrotransposons? Ten different proteins that are necessary for telomere protection have been identified in *Drosophila*. From these, all except one have homologues in human telomeres indicating that, despite its unique organisation, *Drosophila* telomeres conserved most proteins involved in telomere protection or telomere length homeostasis. Whether they play a role on telomere targeting is being analysed. Moreover, finding which are the telomere components that have adapted to the retrotransposon mechanism will help to understand how HeT-A, TART and TAHRE adapted to its cellular role and which changes were necessary to successfully achieve the unusual symbiotic collab-

oration between telomeric retrotransposons and the *Drosophila* genome.

Structural and functional properties of Drosophila ***telomeric heterochromatin***

As is the rest of eukaryotes, *Drosophila* telomeres are heterochromatic and, therefore, genetically silent. However, in *Drosophila*, telomere maintenance relies on the expression of genes located at the actual telomeres. How is their expression regulated? Because of their retrotransposon nature, expression of the telomeric retrotransposons is regulated by the RNAi machinery (reviewed in Casacuberta and Pardue, 2006). Mutations in two different genes of the RNAi pathway, spn-E and aub, regulate the expression of HeT-A and TART in germ cells. Also in organisms with classical telomeres, such as *S. pombe* and Tetrahymena, mutations in the RNAi machinery disrupt telomeric silencing. On the other hand, in *Drosophila*, we have found that mutations in the Jil-1 kinase enhance telomeric silencing. Jil-1 phosphorylates Ser10 at the histone H3 tail, a modification that antagonises methylation of H3K9 and, therefore, HP1 binding and silencing. Does Jil-1 help to maintain expression of the telomeric retrotransposons? How is it recruited to telomeres? Jil-1 is known to interact with regulators of chromatin structure, such as Z4 and chromator. Do they play any role in the regulation of gene expression at *Drosophila* telomeres? We are investigating these questions.

Regulation of homeotic gene expression

In *Drosophila*, the homeotic genes are organised in two clusters, the antenapedia and bithorax complex-

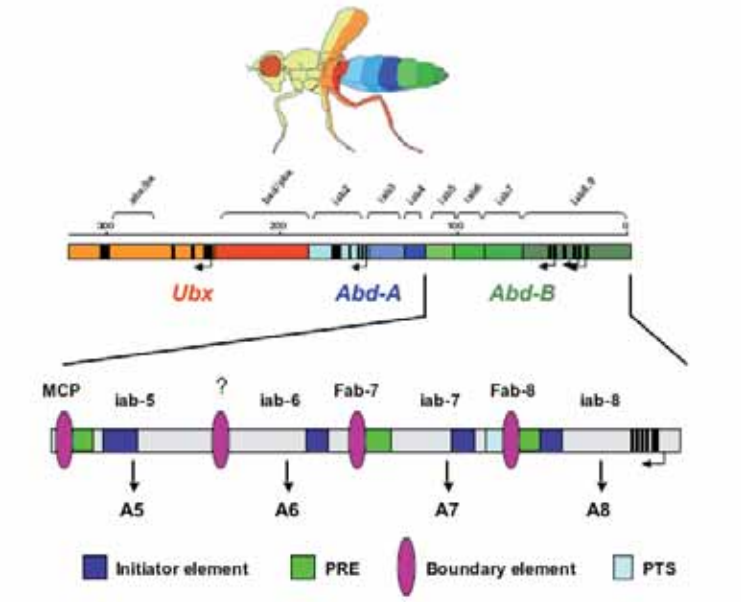

Figure 7. Structural organisation of the bithorax complex (BX-C) of Drosophila.

es. The bithorax complex (BX-C) contains three homeotic genes, Ultrabithorax (ubx), Abdominal-A (Abd-A) and Abdominal-B (Abd-B), which are responsible for specifying the identity of the posterior parasegments of the fly. The expression of these genes is regulated by a complex cis-regulatory region covering 300 kb of DNA divided into nine segment/parasegment-specific subregions in which each domain controls the expression of a single homeotic gene in a particular parasegment. Early in development, *gap* and *pair-rule* genes initiate parasegment-specific patterns of homeotic gene expression which, later in development, are maintained by two ubiquitously expressed groups of proteins, the polycomb group (PcG) and the trithorax group (TrxG), responsible for maintenance of the silenced and activated states respectively. BX-C is organised in various domains, which contain positive and negative regulatory elements, not only to direct the establishment of the expression pattern (IAB initiator elements) but also to maintain it throughout development (PRE regulatory elements). There are also specialised boundary elements that delimit the different regulatory domains. (See Figure 7).

The contribution of chromatin structure and nuclear organisation

Chromatin has an essential contribution to the regulation of homeotic gene expression. Regulatory elements involved either in initiation or maintenance are characterised by a similar pattern of histone modifications but they show a different accessibility to nucleases (Pérez-Lluch *et al*, submitted). At BX-C, IABs are accessible to nucleases only during early embryo development but, on the contrary, PREs are sensitive to nucleases at all stages of embryo development and independently of the silencing activity of the PRE suggesting that these regulatory regions are depleted of nucleosomes in both the ON and OFF transcriptional states. Chromatin-mediated interactions, and nuclear organisation, play an important role in the regulation of homeotic gene expression. We have identified a new PRE in the iab-6 cis-regulatory domain of BX-C and we have shown that it interacts with the Abd-B promoter in cells that do not express the gene (Pérez-Lluch *et al*, submitted). Moreover, 3C analyses showed that different PREs, which regulate the expression of the different homeotic genes of BX-C, are in close proximity to each other within the nucleus indicating the formation of chromatin loops. These loops would be stabilised by chromatin-chromatin interactions between the regulatory elements implicated in maintenance of the silenced state. These results suggest a role of three-dimensional chromatin folding in the mechanism through which PRE-tethered PcG protein complexes act over long distances.

GAGA and dSAP18 contribute to Fab-7 function

One of the best-characterised cis-regulatory elements of BX-C is the Fab-7 element. Fab-7 participates in the regulation of the expression of the homeotic gene Abd-B and contains two different functional elements, a PRE and a boundary region. Fab-7 function requires the contribution of GAGA, a trxG protein previously identified as a transcriptional activator of the homeotic genes (Canudas *et al*, 2005). On the other hand, GAGA interacts with dSAP18, a component of the Sin3/HDAC co-repressor complex (Espinás *et al*, 2000), and this interaction also contributes to the regulation of Fab-7 function (Canudas *et al*, 2005). dSAP18 appears to have a more general contribution to the regulation of gene expression in *Drosophila*. Using *Drosophila* expression microarrays, changes in gene expression in null dsap18 mutant embryos were determined. These analyses show that most of the genes exhibiting altered expression in the mutant background are related to insect immunity. Our preliminary data points to an implication of dSAP18 in the regulation of genes controlled by the Toll pathway (Costa *et al*, in preparation). Moreover, dSAP18 associates to both chromosomes and the nuclear matrix, and forms a complex with the *Drosophila* homologue of pinin, a protein factor involved in mRNA splicing (Costa *et al*, 2006), suggesting a role for dSAP18 in linking RNA processing and chromatin regulation events. On the other hand, GAGA is a general transcriptional regulator that plays multiple functions in *Drosophila*, which are likely regulated through post-translational modifications (Bonet *et al*, 2005).

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RESEARCH NETWORKS AND GRANTS

Cromatina silenciada: análisis de los factores y mecanismos implicados en su formación y mantenimiento
MEC, BFU2006-01627/BMC: 2007-2009
Project Coordinator: Ferran Azorín

Estudio de los telómeros de Drosophila; relación evolutiva y funcional con los telómeros de telomerasa. Los retrotransposones /HeT-A/ y /TART/ y su relación con otros componentes teloméricos
MEC, BFU2006-13934/BMC: 2007-2009
Project Coordinator: Elena Casacuberta

Epigenética: Mecanismos y enfermedad
MEC, CONSOLIDER-INGENIO 2010. CSD2006-49: 2006-2011
Project Coordinator: F. Miguel Beato

Drosophila telomere heterochromatin: Gene silencing and telomere targeting
International Reintegration Grant (IRG)
Marie Curie Action: 2006-2008
Project Coordinator: Elena Casacuberta

Episomal vectors as gene delivery systems for therapeutic application
European Comission FP6-2003-LSH-2: 2005-2008
Project Coordinator: Ferran Azorín

Ànalisi estructural i funcional de la cromatina
CIRIT-Generalitat de Catalunya 2005SGR678: 2005-2008
Project Coordinator: Ferran Azorín

Caracterización estructural y funcional de la cromatina centromérica
MCyT, BMC2003-243: 2003-2006
Project Coordinator: Ferran Azorín

Cromatina y expresión génica: análisis a nivel molecular del silenciamiento de loci eucromáticos
MCyT, BMC2002-905: 2002-2005
Project Coordinator: Mª Lluïsa Espinás

Análisis de mecanismos de regulación génica mediados por traducción de señal en Drosophila
MCyT, GEN2001-4846-C05-03: 2002-2005
Project Coordinator: Jordi Casanova

Characterisation of the role of histone H1 and its post-translational modifications in the functional regulation of chromatin
Marie Curie Reintegration Grants (IRG): 2007-2009
Project coordinator: Alejandro Vaquero

COLLABORATIONS
Determination of the chromosomal distribution of chromatin binding proteins in Drosophila *politene chromosomes*
Sergio Pimpinelli (University of Rome, Italy)

Telomere structure and function in Drosophila
Mary-Lou Pardue (Department of Biology, MIT, USA)

Development of mammalian episomal-vectors
Hans Joachim Lipps (University of Witten, Germany)

Ferran Azorin's group, March 2006.

Signalling in morphogenesis

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The development of multicellular organisms requires changes in cell populations in terms of their proliferation, differentiation, morphology and migration. These synchronised changes are controlled by the genes that specify cell fate and by the capacity of cells to respond to extracellular cues. This is achieved by means of signalling mechanisms that elicit cellular responses that ultimately are responsible for the morphogenetic events that occur during development. Two key steps in these events are the mechanisms that regulate the appropriate spatial and temporal activation of the signalling pathways and the mechanisms that link these pathways with the cell effectors in order to elicit cell responses in terms of gene activity or cell morphology. Our research efforts focus on the study of these phenomena in the context of the whole organism. The basic similarity between developmental processes in different species justifies the choice of an organism as a model system, in our case *Drosophila melanogaster*. In particular, we analyse the above mechanisms in two model systems in this fly, the Torso receptor signalling pathway and the tracheal system morphogenesis.

The *torso-like* gene as a transducer of ovarian positional information for the activation of the Torso receptor in the embryo

To address the control of activation of signalling pathway activation, we analyse how the positional information from one tissue can be used to differentially activate a signalling pathway in another group of cells. In particular, we study the role of the gene *torso-like* (*tsl*) as a transducer of the positional information from the ovary to the embryo. As a consequence of *tsl* activity, the Torso RTK receptor is activated only in embryonic regions that correspond to the areas of the oocyte that had previously been in apposition to the follicle cells expressing the *tsl* gene. (See Figure 1).

We have functionally dissected the *tsl* promoter by means of the analysis of fusions of various fragments of the promoter with the lacZ gene in transgenic flies. We have identified distinct enhancers responsible for the expression of the gene in several populations of follicle cells in the ovary. We are currently using these constructions and their regulation in a number of mutant backgrounds to identify the putative elements that regulate their expression. We have also taken advantage of these enhancers to generate GAL4 lines to examine the functional significance of *tsl* expression in groups of cells, either by crossing them with a UAS-*tsl* line and checking whether they can rescue *tsl* mutants or by crossing them with a UAS-*tslR*NAi line to verify whether they generate *tsl* phenotypes.

Analysis of Capicua as a regulator of gene transcripction by the Torso RTK pathway

Regarding signalling effectors, we study the regulation of the activity of the transcriptional repressor Capicua by the Torso pathway as a link between signalling and gene activity. Specification of the terminal regions of the *Drosophila* embryo depends on the Torso RTK pathway, which triggers expression of the zygotic genes *tailless* (*tll*) and *huckebein* (*hkb*) at the embryonic poles. However, it has been shown that the Torso signalling pathway does not directly activate expression of these zygotic genes; rather, it induces their expression by inactivating, at the embryonic poles, a uniformly distributed repressor activity that involves the Groucho (Gro) co-repressor.

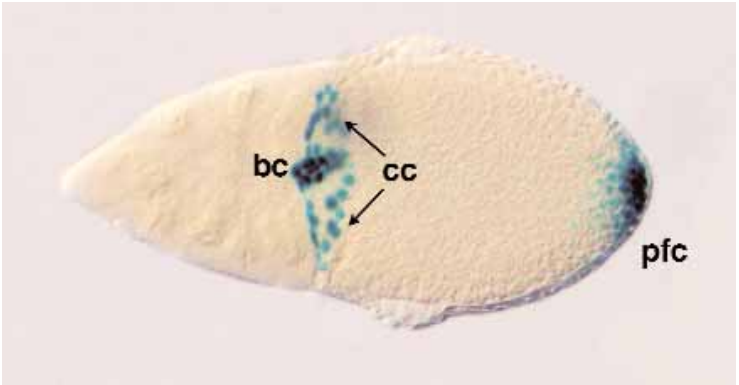

Figure 1. tsl *expression in the eggchamber as visualised by a tsl-lacZ construct. Expression can be detected in the border cells (bc), the posterior follicle cells (pfc) and in the centripetal cells (cc).*

Another component of this co-repressor complex is the product of *capicua* (*cic*), which encodes a putative transcription factor with a DNA-binding domain of the HMG box class and appears to be the target inhibited by the Tor signal. In particular, it has been shown that Torso signalling regulates the accumulation of the Capicua transcriptional repressor: as a consequence of Torso signalling Capicua is down-regulated specifically at the poles of blastoderm stage embryos. Cic activity is not restricted to embryonic terminal patterning as this protein also mediates follicle cell determination and wing vein patterning, thereby acting as a repressor that is down-regulated by the EGFR signalling pathway. Similarly to Gro, Cic has also been conserved in many organisms during evolution, indicating that these two proteins could be part of a conserved repressor complex that is down-regulated by the Ras/Raf molecular cassette in several cellular contexts. By extending the current model, we have shown that activation of the Torso pathway triggers *tailless* expression without eliminating Capicua. While activation of the Tor pathway at the embryonic poles down-regulates Cic, Tor signalling appears to be necessary but not sufficient to eliminate Cic protein, as it can do so only at the embryonic poles. In addition, impairment of Cic repressor function is not an absolute requirement for *tll* expression, as this gene is expressed in situations where the Cic repressor is still functional. In this regard, *tll* expression appears to be the result of a balance between repressor and activator factors and Cic repression might be overcome provided that activation is enhanced. Finally, our analysis has unveiled differences between the terminal and the central embryonic regions that are independent of Torso signalling, hitherto thought to be the only system responsible for conferring terminal specificities.

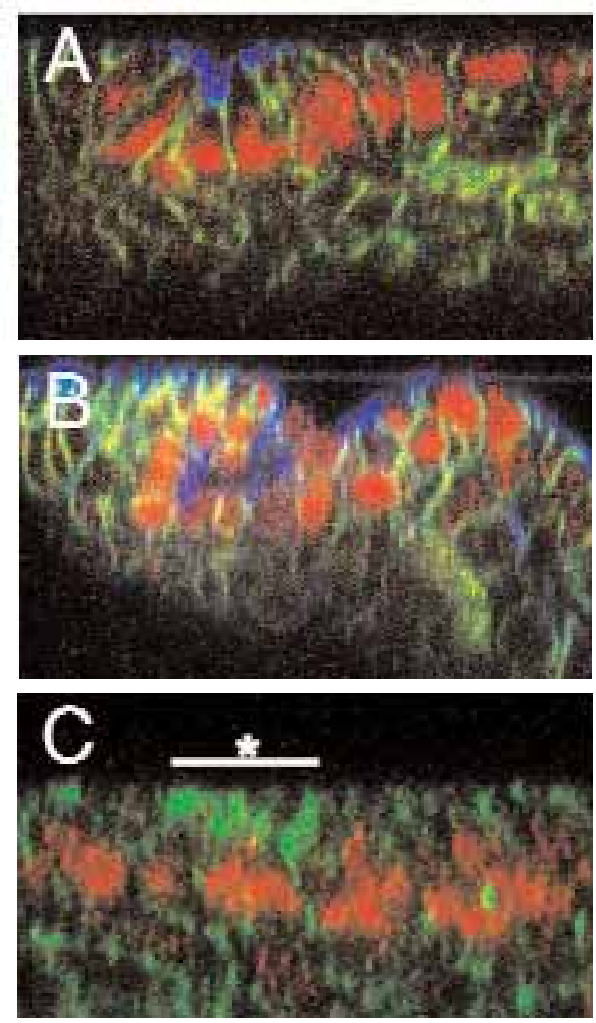

Figure 2. *(A) At the onset of invagination at early stage 11 a small group of cells has reduced their apical perimeter, and the epithelium begins to bend. (B) As invagination proceeds, cells of the dorsal side of the placode have rotated completely around their axis and are found inside the embryo while ventral cells gradually slide beneath, both movements leading to the formation of a finger-like structure. (C) Accumulation of Myosin II is detected at the onset of invagination in the cells initiating apical constriction (white line with an asterisk), as visualised by a GFP-tagged form of Myosin II light chain. In A and B anti-Neurotactin labels the basolateral and basal sides of all epithelial cells (in green), while PKC labels their apical side (in blue). In the three panels tracheal cells were labelled using an anti-trachealess antibody (in red).*

Signalling and cytoskeleton organisation in tracheal morphogenesis

To address the link between signalling and cytoskeleton, we have studied the mechanisms involved in tracheal morphogenesis. To this end, we have begun a detailed description of tracheal morphogenesis at the cellular level in order to analyse the contribution of the different cytoskeleton elements and cell organelles to this process and to identify the changes in these elements that are controlled by signalling pathways and the mechanisms involved in this control.

Tracheal invagination

We have begun to approach this question by studying how a group of cells from an epithelial sheet initiate invagination to ultimately form the *Drosophila* tracheal tubes. The tracheal system of this fly is a complex tubular network that conducts oxygen from exterior to internal tissues. It arises from the tracheal placodes, clusters of ectodermal cells that appear at each side of 10 embryonic segments, from the 2nd thoracic segment to the 8th abdominal segment. Tracheal cells are specified by the activity of a set of genes whose expression in these cells is controlled by the genes that specify positional cues along the embryonic body axes. Cells of each cluster invaginate and migrate in stereotyped and different directions to form each of the primary tracheal branches. The general conclusion from many studies is that the direction of migration of the tracheal cells relies on

a set of positional signals provided by nearby cells. In addition, the establishment of interactions between tracheal cells and their substrates is a crucial step in tracheal cell migration, a process ultimately determined by molecules expressed at their surface. (See Figure 2.)

We have found that tracheal invagination begins by apical constriction in a small group of cells that begin internalisation. This is followed by distinct rearrangements of adjacent cells in the dorsal and ventral part of the placode. We have analysed how this process is regulated by the activity of the trachealess (trh) transcription factor and EGF Receptor (EGFR) signalling. Our results point to a two-step model by which *trh* induces and organises tracheal invagination. First, trh activity appears to outline an invagination field, a region of cells that acquire the capacity to invaginate. A second outcome of *trh* is accomplished by the triggering of EGFR signalling, which leads to the spatial and temporal organisation of tracheal invagination. It is the activity of the EGFR pathway that converts the tracheal cell potential to invaginate into the organised process that results in a "three-layer organisation" and initiation of tube formation. We have also found that the spalt (sal) transcription factor downregulates EGFR signalling in the dorsal side of the tracheal placode, and that this modulation of EGFR signalling is required for the organised invagination of tracheal cells. We have determined that tracheal invagination is associated with a distinct recruitment of Myosin II to the apical surface in the cells of the invaginating edge and that Myosin II is required for the proper invagination of tracheal cells. We have identified *crossveinless-c* (*cv-c*), a gene coding for a RhoGTPase Activating Protein (RhoGAP), as a key intermediate in this process. Taken together, our results unveil a developmental pathway, linking genes specifying cell fate and signalling pathways with cytoskeleton modifications that underlie early remodelling of tracheal cell shape. (See Figure 3.)

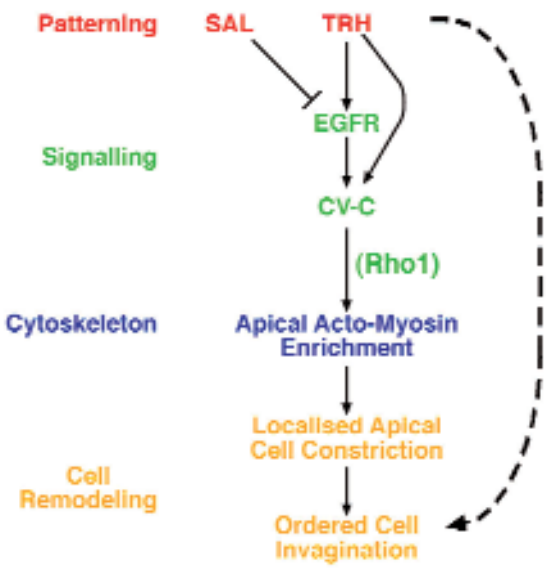

Figure 3. Model for tracheal cell invagination.

Chitin and glycans in tracheal morphogenesis

By genetic screening, we have identified new alleles involved in trachael development, which belong to the same complementation group as the previously identified cystic (cyst) and mummy (mmy) mutations. More precisely, *cyst* was originally singled out as a gene required for the regulation of tracheal tube diameter. In collaboration with Guy Tear's lab, we have shown that all these are mutants of a gene encoding the UDP-N-acetylglucosamine diphosphorylase, an enzyme responsible for the production of UDP-N-acetylglucosamine, which is essential for chitin synthesis, membrane biosynthesis, protein N- and O-glycosylation, and GPI anchor biosynthesis. We have characterised the *cyst/mmy* tracheal phenotype and after histological examination we concluded that *mmy* mutant embryos lack chitin-containing structures, such as the procuticle at the epidermis and the taenidial folds in the tracheal lumen. While most of the tracheal morphogenetic defects in these mutants can be attributed to the lack of chitin, when compared to *krotzkopf verkehrt* (*kkv*) chitin-synthase mutants, *mmy* mutants show a stronger phenotype indicating that some of the *mmy* phenotypes are chitin-independent. An additional lack of GlcNAc-containing proteins at the cell surface or within the extracellular matrix could further affect luminal stability in *mmy* embryos. Furthermore, tracheal defects in *mmy* phenotypes could partly arise as a result of the impairment of a signalling process mediated by GlcNAc-containing proteins, as GlcNAc is a major component of glucosaminoglycan chains attached to heparan sulphate proteoglycans (HSPGs), which play a major role in multiple signalling pathways. (See Figure 4.)

A remarkable feature of the dorsal trunk of *mmy* embryos is the absence of taenidial folds, the annular rings around the tracheal lumen. As these structures are believed to provide a degree of stiffness to tracheal tubes, their absence could play a role in the irregular diameter of the dorsal trunk. This study has led us to propose that tracheal tube size may be a structural property of the organisation of each specific branch.

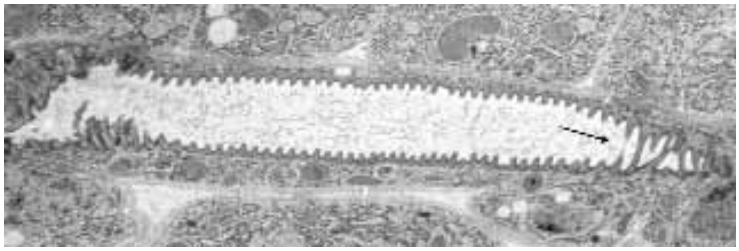

Figure 4. Electron micrograph of a longitudinal section of a wild-type tracheal dorsal trunk showing the lumenal structure and tracheal cells. The section is not in the middle of the dorsal trunk but at a more saggital point, showing a tube that is not cut at its greatest width; arrow points to the areas where the cut is most saggital and the taenidial folds are seen closest to the luminal membrane, showing their characteristic ridges (picture taken at the Electron Microscopy facilities of the Universitat de Barcelona).

Association of tracheal placodes with leg primordia in *Drosophila* and implications for the origin of insect tracheal systems

Adaptation to diverse habitats has prompted the development of several organs in a number of animals to better exploit their living conditions. This is the case of the respiratory organs of arthropods, which range from tracheae in terrestrial insects to gills in aquatic crustaceans. While tracheal development in *Drosophila* has been studied extensively, the origin of the tracheal system remains a mystery. In collaboration with Michalis Averof, we have shown that tracheal placodes and leg primordia in *Drosophila* arise from a common pool of cells, with differences in their fate controlled by the activation state of the *wingless* signalling pathway. We have also elucidated early events that trigger leg specification and shown that cryptic appendage primordia are associated with tracheal placodes even in abdominal segments. The association between tracheal and appendage primordia in *Drosophila* is reminiscent of the association between gills and appendages in crustaceans. To further examine these similarities, we have cloned homologues of the tracheal inducer genes and studied their expression patterns in two divergent groups of crustaceans. We have found that homologues of tracheal inducer genes are specifically expressed in the gills of crustaceans. Therefore, crustacean gills and insect tracheae, hitherto considered independent systems for gas exchange, may share a number of features in their developmental origin and specification. This hypothesis raises the possibility of an evolutionary relationship between these structures.

PUBLICATIONS

Araujo SJ, Aslam H, Tear G and Casanova J (2005) *mummy/cystic* encodes for an enzyme required for chitin and glycan synthesis, involved in trachea, embryonic cuticle and CNS development - Analysis of its role in *Drosophila* tracheal morphogenesis. Dev Biol, 288:179-193

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RESEARCH NETWORKS AND GRANTS

Análisis de mecanismos de regulación génica mediados por transducción de señal en Drosophila (GEN2001-4846-C05-03)
Ministerio de Ciencia y Tecnología: 2002-2005
Project coordinator: Jordi Casanova

Estudio de mecanismos de transducción de señal y de migración celular en el desarrollo embrionario de Drosophila (BMC2003-00230)
Ministerio de Ciencia y Tecnología: 2003-2006
Project coordinator: Jordi Casanova

Ajut per a Grups de Recerca Singular de la Generalitat de Catalunya (SGR2005-00508)
Departament d'Educació i Universitats: 2005-2008
Project Coordinator: Jordi Casanova

COLLABORATIONS

Guy Tear (MRC Centre for Developmental Neurobiology, UK)

Michalis Averof (IMBB, Greece)

Growth factor structure
Miquel Coll (IRB Barcelona, Spain)

Jordi Casanova's group, March 2006.

Cell division group

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Cayetano González

Our goal is to understand the mechanisms of cell division. For this purpose, we use a multidisciplinary approach that combines genetics, molecular biology and advanced *in vivo* microscopy. We use *Drosophila* as well as cultured cells derived from vertebrates as model systems. Current on-going projects include the study of the mechanisms of spindle assembly, the characterisation of new centrosomal proteins and the modelling of cancer in *Drosophila*.

Spindle assembly *in vivo*
Direct visualisation is becoming mandatory to unravel the complex processes that occur within the living cell. Using protocols developed in our lab we are obtaining new information on the behaviour of specific proteins labelled with florescent tags (Rebollo and González 2000; Lange *et al*, 2002; Sampaio *et al*, 2001; Rebollo et al, 2004).

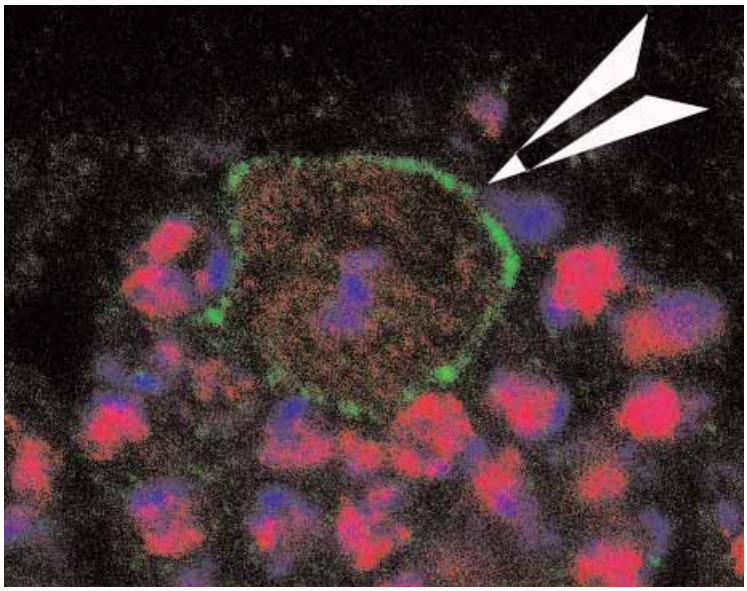

Figure 1. *Mutant neuroblast (NB) undergoing symmetric divisions. The pointed NB (nervous system stem cell) is undergoing mitosis. Miranda, a cell polarity protein is stained in green, the centrosomal protein CP190 is shown in red and DNA in blue. The smaller cells are ganglion mother cells derived from successful asymmetric division of other NBs. In the mutant NB there are no centrioles thus CP190 is not localised in two foci but is distributed throughout the cell and Miranda is inappropriately localised around the entire cortex rather than being localised basally as it should be in asymmetrically dividing NBs.*

Modelling cancer in *Drosophila*
We are starting to exploit *Drosophila* to study basic principles regarding cell proliferation and malignant growth (Caussinus and González, 2005; Wodarz and González, 2006). Loss of cell polarity and cancer are tightly correlated, but proof of a causative relationship remains elusive. In stem cells, loss of polarity and impairment of asymmetric cell division could alter cell fate and thereby render daughter cells unable to respond to the mechanisms that control proliferation. To test this hypothesis, we have developed a *Drosophila* model of tumour transplantation using larval neuroblasts (NBs) containing mutations in various genes that control asymmetric cell division.

Molecular analysis of centrosomes
We have recently cloned the gene that encodes a centriolar protein. We have mutant alleles, functional GFP fusions and antibodies that will be instrumental in the molecular dissection of this organelle (Lange *et al*, 2002; Tavosanis and González, 2003).

Protein traps
The specific localisation of proteins in different compartments is a key aspect of cell division. We have now automated a protein trap protocol (González and Bejarano, 2000; Isalan *et al*, 2005; Santori *et al*, 2006) which is being applied in several high-content screens.

Peter Askjaer's work
Peter Askjaer works as an independent Ramón y Cajal researcher within the lab. Peter's group uses the nematode *Caenorhabditis elegans* to analyse processes of cell and developmental biology with an emphasis on the nuclear envelope.

PUBLICATIONS

Caussinus E and González C (2005) Induction of tumour growth by altered stem-cell asymmetric division in *Drosophila*. Nat Genet, 37:1125-1129

Franz C, Askjaer P, Antonin W, Iglesias CL, Haselmann U, Schelder M, de Mario A, Wilm M, Antony C and Mattaj IW (2005) Nup155 regulates nuclear envelope and nuclear pore complex formation in nematodes and vertebrates. EMBO J, 24:3519-3531

Furnes C, Arnesen T, Askjaer P, Kjems J and Szilvay AM (2005) HIV-1 Rev oligomerisation: activity restoration of a defect mutant by addition on an extra basic domain. Retrovirol, 2:39

Isalan M, Santori MI, González C and Serrano L (2005) Localized transfection on PCR-coated magnetic arrays. Nat. Methods, 2:113-118

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Santori, MI, González C, Serrano L and Isalan M (2006)Transfection with magnetic beads coated with PCR products and other nucleic acids. Nat Protocols, 1:526-531

Schetter A, Askjaer P, Piano F, Mattaj IW and Kemphues K (2006) Nucleoporins NPP-1, NPP-3, NPP-4, NPP-11 and NPP-13 are required for proper spindle orientation in *C. elegans*. Dev Biol, 289:360-371

Wodarz A and González C (2006) Connecting cancer to the asymmetric division of stem cells. Cell, 124:1121-1123

RESEARCH NETWORKS AND GRANTS

Regulación de la localización subcelular y su contribución a la transformación cancerosa
Spanish MEC: 2004-2006
Principal Investigator: Cayetano González

Molecular analysis of Drosophila *cell division, MADCDC*
EU V Framework Programme
Research Network Contract: 2003-2007
Project Coordinator: M Gatti

Integrative approach to cellular signalling and control processes: bringing computational biology to the bench, COMBIO
EU VI Framework Programme, STREP: 2004-2007
Project Coordinator: Luis Serrano

Alteraciones en la localización subcelular y transformación cancerosa: Determinación de su utilidad diagnóstica
Fundación Médica Mutua Madrileña Automivilistica: 2004-2007
Principal Investigator: Cayetano González

Generation of cancer tumour models in Drosophila
Generalitat de Catalunya: 2006-2009
Principal Investigator: Cayetano González

Cancer stem cells and asymmetric division, ONCASYM
EU VI Framework Programme, STREP: 2006-2009
Project Coordinator: Marcos González Gaitan

Centrosome 3D: Hacia la comprensión estructural y funcional del centrosoma
CONSOLIDER - INGENIO 2010: 2006-2010
Project Coordinator: Luis Serrano

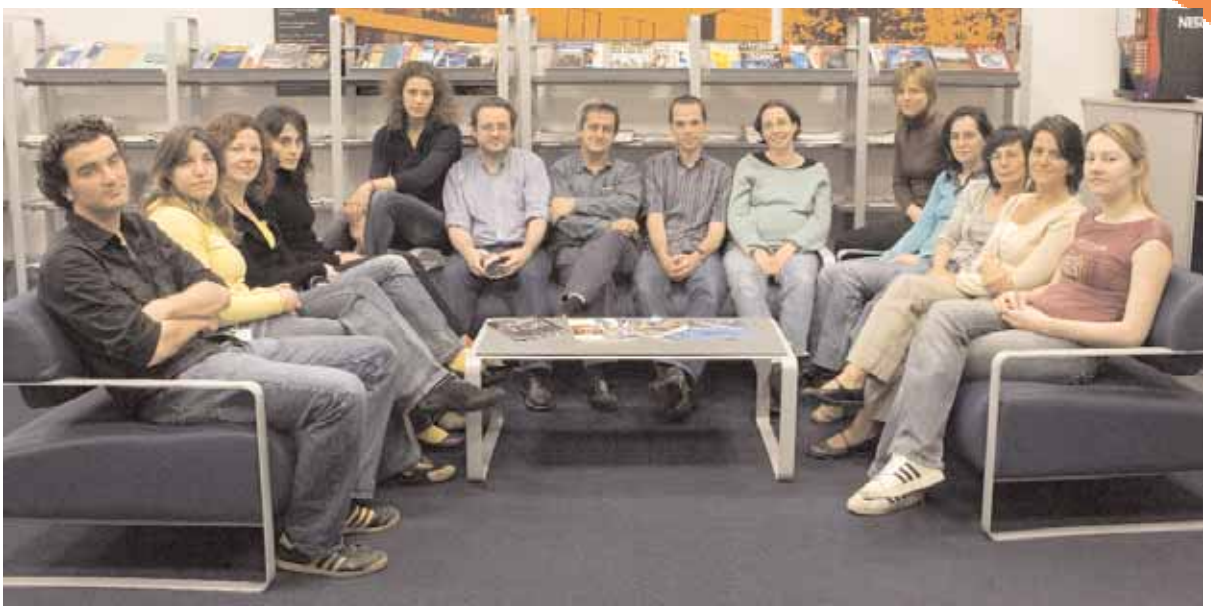
Identification of pathways that are relevant for the malignant transformation of stem cells in *Drosophila*.
Spanish MEC: 2006-2009
Principal Investigator: Cayetano González

OTHER FUNDING SOURCES

Sponsored research agreement with CIBASA: 2006-2008

AWARDS

Conference Award from the Institucio Catalana de Recerca i Estudis Avançats (ICREA) for the organisation of the international meeting: "*Drosophila* as a model for human diseases" at IRB Barcelona, October 5-7, 2006



Cayetano González's group, March 2006.

Drosophila limb development

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Research Assistant
Lidia Pérez

Marco Milán

A central question in modern developmental biology is how the growth and patterning of tissues are controlled at a molecular and genetic level. *Drosophila melanogaster* provides an excellent model system to approach this question because of its suitability for genetic and molecular manipulation and its well-described developmental biology. Systematic genetic screens for loss-of-function mutations, gain-of-function phenotypes and the detection of enhancers have revealed many of the genes involved in a number of developmentally important processes. In addition, the completion of the genomic sequencing project for *Drosophila* allows the use of reverse genetic approaches such as RNA-mediated interference and targeted gene disruption as well as genome-wide expression analyses in order to address a wide variety of questions concerning the developmental biology of the fruitfly.

During the development of multicellular organisms, groups of cells assemble to form tissues that are initially homogenous. The elaboration of spatial pattern often begins by subdivision of the field of cells into smaller territories. The imaginal discs of *Drosophila* provide well-characterised experimental systems in which subdivision of the tissue depends on mechanisms that limit cell mixing to produce stable boundaries. These stable subdivisions are called compartments. In the imaginal discs compartment boundaries serve as signalling centres. Short-range interactions between cells in adjacent compartments induce the expression of the signalling proteins Wingless (Wg) and Decapentaplegic (Dpp) in cells adjacent to the compartment boundaries. Wg and Dpp form long-range extracellular protein gradients centered on the compartment boundaries. Stable boundaries between compartments result in tightly localised sources of these signalling proteins. Intermingling of cells at the boundary causes disorganisation of the signalling centre with disastrous consequences for patterning and growth control.

The *Drosophila* wing imaginal disc is a monolayered epidermal sac. The wing primordium arises from the embryonic ectoderm as a group of around 30 cells that proliferate extensively during larval development to achieve a final size of about 50,000 cells. The wing primordium is subdivided into an anterior (A) and a posterior (P) compartment by the restricted expression and activity of the homeodomain protein Engrailed in P cells. This subdivision is inherited from the embryonic ectoderm. When the wing primordium consists of around 100 cells, it subdivides again into a dorsal (D) and a ventral (V) compartment by the restricted expression and activity of the LIM-homeodomain protein Apterous in D cells. Our research activities seek to elucidate how compartment boundaries are generated, how expression of the organising molecules Wg and Dpp is induced at the these boundaries and how growth and patterning is organised by the activity of Wg and Dpp.

Hedgehog restricts its own expression domain in the *Drosophila* wing

Stable subdivision of *Drosophila* limbs into an anterior (A) and posterior (P) compartment is a consequence of asymmetric signalling by Hedgehog (Hh) from P to A cells. The activity of the homeodomain protein Engrailed in P cells helps to generate this asymmetry by inducing expression of Hh in the P compartment and at the same time repressing the expression of the essential downstream component of the Hh pathway *Cubitus interruptus* (Ci). Ci is a transcription factor, which, in the absence of Hh signalling is converted to a repressor form (Ci^{rep}). Ci^{rep} represses *hh* in A cells. The transcriptional co-repressor Groucho (Gro) also represses *hh* expression in A cells, thus helping to maintain the asymmetry. Gro is ubiquitously expressed but is required only in A cells that receive the Hh signal.

During the last two years, Fernando Bejarano and Lidia Pérez, in collaboration with Christos Delidakis' group in Heraklion (Crete) have analysed the role of

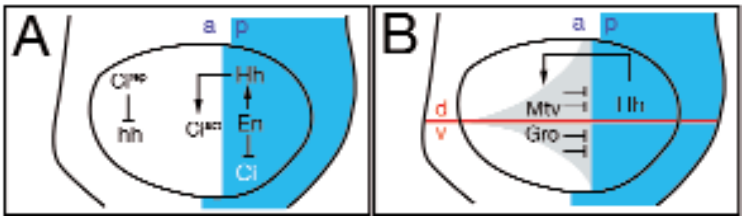

Figure 1. Two mechanisms to repress hh *expression in A cells.*

Groucho in this process. They have presented evidence that Gro exerts this action by binding to the product of *master* of *thickveins* (*mtv*), a target of Hh activity encoding a nuclear zinc finger protein. Two mechanisms are then used to repress *hh* expression in A cells (Figure 1). The first is based on Ci^{rep} acting mainly in those cells not receiving the Hh signal. The second one is based on Hh restricting its own expression domain through the activity of its target gene *mtv*. Fernando and Lidia have provided evidence that these two mechanisms are independent.

Many signalling molecules have been shown to restrict their own expression domains (*ie*, Wingless or Notch). To our knowledge, these results show for the first time that Hh is also involved in restricting its expression domain.

Mutually exclusive domains of Notch and Wingless activities confer stability to the DV boundary in the *Drosophila* wing

Gene regulatory networks in developing organisms have been conserved during evolution. The *Drosophila* wing and the vertebrate hindbrain share the gene network involved in the establishment of the boundary between dorsal (D) and ventral (V) compartments in the wing and adjacent rhombomeres in the hindbrain (Figure 2). The activation of the receptor Notch at the compartment boundaries, due to the activity of the Notch ligands in nearby cells, induces the expression of the signalling molecules Wingless (Wg) and Wnt-1 in boundary cells of the fly wing and the vertebrate hindbrain, respectively (Figure 2). Wg or Wnt-1 maintain the expression of Notch ligands in neighboring cells, thus establishing positive feedback and ensuring high activity of Notch at the compartment boundaries. Notch activity then regulates growth of the surrounding non-boundary cells and is required for maintaining the lineage restriction boundary.

By means of a systems biology approach that combines *in silico* and *in vivo* experiments, during the last two years, Héctor Herranz, in collaboration with Javier Buceta's group at the Parc Científic de Barcelona, have generated a regulatory network for the establishment and maintenance of the DV boundary in the *Drosophila* wing. This regulatory network shows how short-range cell interactions, mediated by the receptor Notch and its ligands, together with long-range cell interactions, mediated by the Wg signalling molecule, shape the boundary and produce the gene expression pattern that is observed *in vivo*. Their data also present *in vivo* and *in silico* evidence that a novel property, conferred by the activity of Notch in boundary cells and mediated by its target gene Cut, is required for the formation of a stable DV boundary: refractoriness to the Wg signalling molecule. Concepts like spatially refined and polarised Notch signalling have been addressed and explained by invoking this property. A robustness analysis of the regulatory network by means of *in silico* experiments complements their results and ensures the biological plausibility of the developmental mechanism proposed.

Finally, we wish to place our conclusions into a broader context. Boundary formation between adjacent rhombomeres in vertebrates relies on the same Wnt/Notch-dependent regulatory network as shown by David Wilkinson's group in the UK. We therefore speculate that boundary cells also need to be refractory to the Wnt signal to generate stable boundaries. We conclude that the robustness and stability of this network, in which the interconnectivity of the elements is crucial and even more important than the value of the parameters used, might explain its use in boundary formation in other multicellular organisms.

Self-refinement of Notch activity through the transmembrane protein Crumbs: Modulation of γ-secretase activity

Cell interactions mediated by Notch-family receptors have been implicated in the specification of tissue boundaries. Tightly localised activation of Notch is critical for the formation of sharp boundaries. In the *Drosophila* wing imaginal disc, Notch receptor is expressed in all cells. However, Notch activity is limited to a narrow stripe of cells along the DV compartment boundary, where it induces the expression of target genes. How a widely expressed protein becomes tightly regulated at the DV boundary in the *Drosophila* wing is not completely understood.

Over the last two years, Héctor Herranz, in collaboration with the groups led by Fabián Feiguin in Italy and Michalis Averof in Heraklion (Crete) have analysed the role of *crumbs* in DV boundary formation in the wing primordium. Their data indicate that the transmembrane protein Crumbs is involved in a feedback mechanism used by Notch to refine its own activation domain at the *Drosophila* wing margin (Figure 3). Crumbs is a target of Notch in the wing and is

required to reduce the activity of the γ-Secretase complex, which mediates the proteolytic intracellular processing of Notch. Crumbs exerts its function through its large extracellular part, which contains 29 EGF repeats.

Signalling centres along compartment boundaries are required to organise the growth and pattern of the surrounding tissue. However, too much of a signal has deleterious effects. The Notch signalling centre organises growth and pattern of the developing wing primordium partially through the secreted protein Wingless. Wingless activity contributes to limit Notch activity to cells immediately adjacent to the DV boundary. Our data indicate that Notch also contributes to the refinement of its activation domain through its target gene *crumbs*. Crumbs attenuates Notch signalling by repressing the activity of the γ-Secretase complex. Many loss-of-function mutations in the human homologue of Crumbs, CRB1, cause recessive retinal dystrophies, including *retinitis pigmentosa*. Given that the γ-Secretase complex also mediates the intracellular cleavage of the transmembrane protein APP, leading to accumulation of the ABeta peptide in plaques in Alzheimer disease (AD), we postulate that Crumbs is also involved in modulating AD pathogenesis. Our analysis reveals a function for the extracellular part of the Crb protein in this process. It is interesting to note that many mutations that give rise to retinal dystrophies are missense that affect EGF or LG domains of CRB1 Thus, molecular interactions mediated by the extracellular domain of Crb may be critical in both *retinitis pigmentosa* and AD.

Growth control in the proliferative region of the *Drosophila* eye-head primordium: *elbow-noc* gene complex

Notch signalling is involved in cell differentiation and patterning, as well as in the regulation of growth and cell survival. Notch activation at the DV boundary of the *Drosophila* eye-head primordium leads to the expression of the secreted protein Unpaired, a ligand of the JAK-STAT pathway that induces cell proliferation in undifferentiated tissue.

During the last three years, Carlos Luque has focused on the role of the zinc-finger proteins encoded by *elbow* and *no ocelli* in the control of growth of the eye-head undifferentiated tissue. His results show that *elbow* and *no ocelli* are expressed in the highly proliferative region of the eye-head primordium and loss of *elbow* and *no ocelli* activities induces overgrowths of the head capsule, without inducing Upd expression *de novo*. These overgrowths depend on Notch activity indicating that *elbow* and *noc* repress an Upd-independent role of Notch in driving cell proliferation. When the size of the overgrown tissue is increased, ectopic antenna and eye structures can be found.

The observation that increased size of the eye-head primordium leads to ectopic eye and antenna structures suggests that tight regulation of the size of this organ by *elbow* and *no ocelli* is crucial for proper fate specification and generation of the adult structures. We propose a model to explain how de-regulated proliferation produces tissue overgrowth that undergoes fate re-specification depending on the availability of nearby sources of organising molecules. A similar model has already been proposed by the groups of Mlodzik and Pignoni in the US to explain how field size is coupled to fate specification. (See Figure 4.)

Calderón encodes an organic cation transporter of the major facilitator fuperfamily required for cell growth and proliferation of *Drosophila* tissues

The adaptation of growth in response to dietary changes is essential for the normal development of all organisms. The Insulin Receptor (InR) signalling pathway controls growth and metabolism in response to nutrient availability. Although the elements of this pathway have been described, little is known about

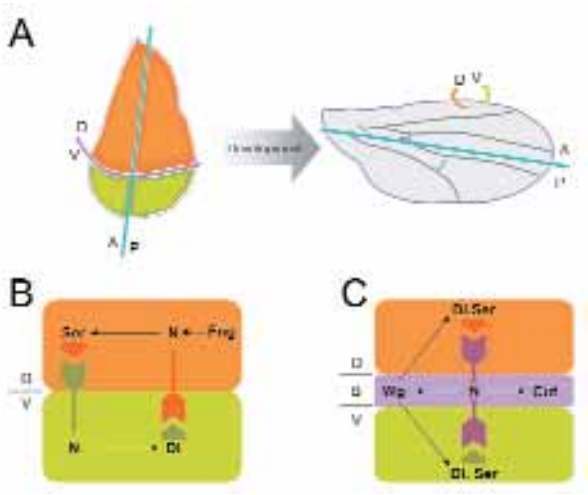

Figure 2. Short-range and long-range cell interactions in DV boundary formation.

Figure 3. Expression of Crumbs at the DV boundary of the Drosophila *wing primordium.*

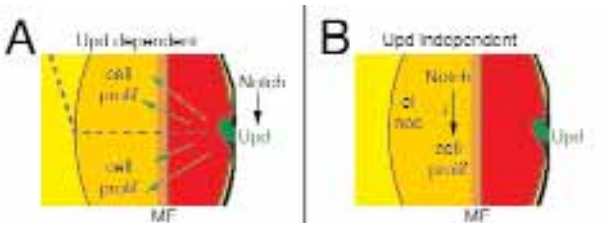

Figure 4. Upd-dependent and independent roles of Notch in inducing cell proliferation in the eye primordium.

the downstream elements regulated by this cascade. Héctor Herranz identified the *calderón* gene while he was a postdoc in Ginés Morata's lab in Madrid. He started its functional characterisation in Madrid and finished it in our lab. *calderón* encodes a protein with highest homology with Organic Cation Transporters of the Major Facilitator Superfamily. His results show that this gene is a new transcriptional target of the InR pathway. These transporters are believed to function mainly in the uptake of sugars, as well as of other organic metabolites. Genetic experiments demonstrate that *calderón* is required cell autonomously and downstream of the InR pathway for normal growth and proliferation of larval tissues. Loss of *calderón* activity mimics the phenotype of mutations in the InR pathway during embryonic and adult development. Expression of *calderón* is positively regulated by the InR downstream effectors PI3 kinase/Dp110 and TOR and its activity is required for TOR-mediated growth induction. Thus, *calderón* is a target of the PI3 kinase/TOR branch of the InR pathway required cell autonomously for insulin-mediated cell growth.

Our results indicate that the growth of imaginal cells may then be modulated by two distinct, but coordinated, nutrient-sensing mechanisms: one cell-autonomous and the other humoral.

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Herranz H and Milán M (2006) Notch and affinity boundaries in *Drosophila*. Bioessays, 28:1-4

Herranz H, Morata G and Milán M (2006) Calderón encodes an organic cation transporter of the major facilitator superfamily required for cell growth and proliferation of *Drosophila* tissues. Development, 133:2617-2625

Herranz H, Stamataki E, Feiguin F and Milán M (2006) Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of γ-secretase activity. EMBO Rep, 7:297-302

Luque CM and Milán M (2007) Growth control in the proliferative region of the *Drosophila* eye-head primordium: the *elbow-noc* gene complex. Dev Biol, 301:327-339

Milán M (2007) Sculpting a fly leg: BMP boundaries and cell death. Nature Cell Biology, 9:17-18

RESEARCH NETWORKS AND GRANTS

Abnormal proteins in the pathogenesis of neurodegenerative disorders (APOPIS)
European Grant, EU-Commission FP6-2002-Lifescihealth-LSH-2002-2.1.3.3: 2004-2006
Project Coordinator: Carlos G. Dotti

Abnormal proteins in the pathogenesis of neurodegenerative disorders
Complementary grant to the European Grant BFU2004-0142-E: 2004-2006
Project Coordinator: Carlos G. Dotti

Genome re-modelling in evolution: functional annotation of segmental gene duplications in Drosophila *and other invertebrates*
BBVA grant: 2004-2005
Project Coordinator: Miguel Manzanares

Afinidades celulares en el desarrollo de organismos muticelulares: el borde de afinidad dorsal-ventral del ala de Drosophila
BFU2004-00167/BMC
Ministerio de Educación y Ciencia: 2004-2007
Project Coordinator: Marco Milán

Generalitat de Catalunya Junior Group Leader Grant (2005 SGR 00118) Spain: 2006-2009
Project Coordinator: Marco Milán

COLLABORATIONS

Modulation of Gamma-Secretase activity in Drosophila
Fabián Feiguin (Cavalieri Ottolenghi Scientific Institute, University of Turin, Italy)

The role of Mtv and Groucho in the repression of hh in the Drosophila *wing*
Christos Delidakis (Institute of Molecular Biology and Biotechnology, FoRTH, and Department of Biology, University of Crete, Heraklion, Greece)

The role of Crumbs in DV boundary formation
Michalis Averof (Institute of Molecular Biology and Biotechnology, FoRTH, and Department of Biology, University of Crete, Greece)

In silico *modelling of DV boundary formation in the* Drosophila *wing*
Javier Buceta, Ramon Reiguada and Frances Sahgues (Centre Especial de Recerca en Química Teòrica,

CeRQT, Parc Científic de Barcelona, Spain)
Ramon Reiguada and Frances Sagues (Departament de
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Marco Milán's group, March 2006.

Exploring the cellular network linked to protein synthesis

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Ana Catarina Gomes (Portugal)

Lluís Ribas de Pouplana

We study the connections between the protein synthesis machinery and the rest of the cellular environment, mainly in human protozoan parasites (Figure 1). Gene expression and protein synthesis constitute the central core of molecular biology and, as such, are tightly linked to the networks that regulate cells and tissues. The sophisticated machinery required to translate genes is now well characterised, and this body of knowledge offers the possibility to investigate how the components for genetic code translation influence and integrate themselves with the rest of the cellular metabolism. We focus mainly on the links between gene expression and pathogenicity in human pathogens. Our activities also address organelle-specific protein synthesis and the evolution of the eukaryotic cell through the analysis of gene translation apparatus.

Gene expression and parasite pathogenicity

We examine the connections between infectious processes in humans and the protein synthesis apparatus of the pathogens involved. Thomas Jones is characterising an unusual aminoacyl-tRNA synthetase in the human intracellular parasite *Mycoplasma penetrans*. This parasite presents remarkable genome reduction. However, some of the components of its protein synthesis machinery display new domains of unknown function. We have shown that these modified enzymes are better discriminating catalysts than their *E. coli* homologues. We are now examining how this improvement in substrate recognition is achieved (Jones, Alexander and Ribas de Pouplana, in preparation).

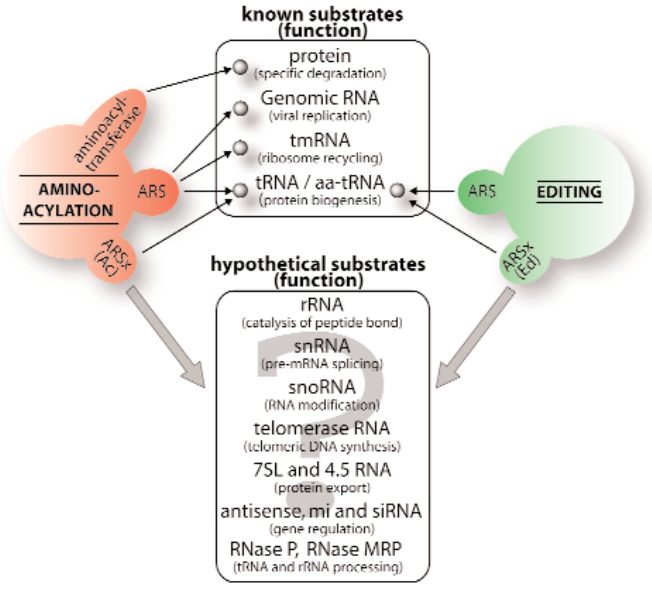

Figure 1. Proposed links between the RNA aminoacylation and deacylation reactions and several cellular pathways unrelated to protein synthesis (Geslain and Ribas de Pouplana, 2004).

Our research also addresses the genome dynamics and the functional role of a family of inflammation-activator domains that are transferred between different protein synthesis enzymes through a ubiquitous process of domain shuffling that is not understood. In the human pathogen *Entamoeba histolytica*, one of these domains doubles its genomic dose through its selective integration in two unrelated enzymes. Manuel Castro is testing the hypothesis that this domain is involved in the induction of local inflammation in the human gut. We are also studying the mechanisms of domain integration involved in this process, and the effect of the newly incorporated domain in the activity of its receiving enzyme (Figure 1).

Finally, the incorporation of Alfred Cortes (ICREA Jr) to the group will allow us to start a new line of research into the mechanisms of gene translation regulation in *Plasmodium falciparum*. This parasite is the main causal agent of malaria, and is responsible for over a million deaths a year. Using a multigenic family of proteins that display differential expression in clonal lines of this parasite, we will examine the

role of chromatin structure and modification in the control of gene expression in *Plasmodium*.

Organelle protein synthesis

We are currently studying the cellular mechanisms that control and coordinate protein synthesis in the cytoplasm and the lumen of cellular organelles, like mitochondria. Most of the proteins required for protein synthesis in the mitochondria are nuclear-encoded, and need to be imported to this organelle. In contrast, most species have maintained their mitochondrial tRNA genes in the mitochondrial genome. Interestingly, several mitochondrial tRNA aminoacylation enzymes are organelle-specific. What prevents the eukaryotic cell from reducing the complexity of its protein synthesis apparatus and from using the same aminoacyl-tRNA synthetases (ARS) in the nucleus and the mitochondria?

To answer these questions we are now examining tRNA aminoacylation in two extreme cases: *Trypanosoma brucei* lysyl-tRNA synthetase (TbKRS) and *Drosophila melanogaster* seryl-tRNA synthetase (DmSRS). *Trypanosoma* are an exception among eukaryotes because their mitochondrial genome does not contain tRNAs. All the tRNAs used in mitochondrial gene expression in *Trypanosoma* are imported from the nucleus. However, some of these tRNAs are still aminoacylated by mitochondria-specific ARS, which also need to be imported. Yaiza Español is currently characterising the mitochondrial TbKRS and comparing it with its cytoplasmic counterpart. Our results indicate that specific mitochondrial modifications may contribute to the maintenance of this complex mechanism. Specific mitochondrial modifications could be related to characteristics of the mitochondrial ribosome and translational apparatus or be indirectly linked to more general nucleic acid modification strategies that occur in *Trypanosoma* organelles.

An equally fascinating case is that of *Drosophila melanogaster*, whose genome apparently codes for two forms of mitochondrial SRS, whose temporal expression seems to be mutually exclusive. Tanit Guitart is currently investigating these enzymes and the reasons behind their unusual distribution.

New methods for the discovery of protein synthesis inhibitors

Major research effort is devoted to the search for improved methods of antibiotic discovery. To this end we are characterising aminoacyl-tRNA synthetases from pathogenic organisms and developing new cellular tools for their expression in human cells (see Geslain *et al*, 2006; Figure 2). Renaud Geslain is studying the mechanisms of recognition that control the correct aminoacylation of $tRNA^{Ser}$ in *Trypanosoma*. Using genetic screens in yeast, he is also developing new tools for the manipulation of ARS structures and the modulation of their substrate specificities. Complementarily, Teresa Bori-Sanz is developing new human cell lines that will express modified ARSs from pathogenic organisms. Our final goal is to develop a new screening procedure for molecular inhibitors of these enzymes.

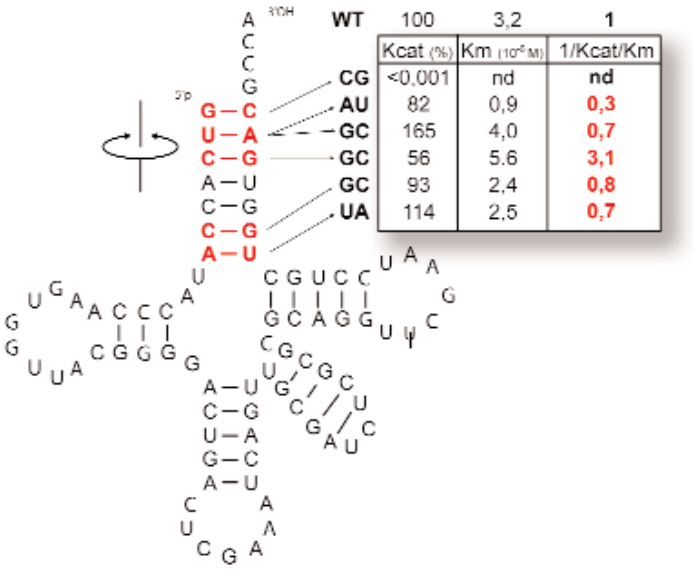

***Figure 2**. The characterisation of the recognition of tRNASer by Trypanosoma seryl-tRNA synthetase (Geslain* et al*, 2006).*

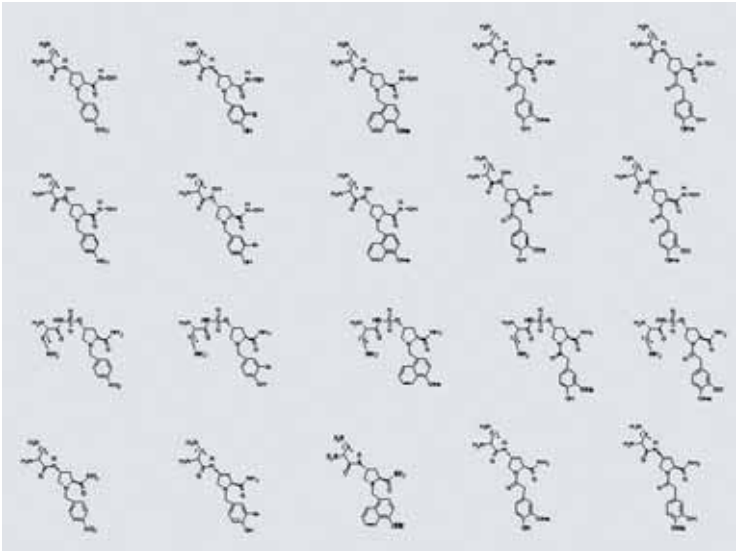

***Figure 3**. Some components of a combinatorial library based on analogues of ARS reaction intermediates (Farrera* et al*, in preparation).*

This project is done in collaboration with the Combinatorial Chemistry Unit of the Barcelona Science Park, which provides us with *ad hoc* chemical libraries designed to bind and inhibit the active sites of aminoacyl-tRNA synthetases (Farrera *et al*, in preparation; see Figure 3).

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RESEARCH NETWORKS AND GRANTS

Desarrollo de un nuevo método para la selección de antibióticos
Spanish Ministry of Science and Technology grant BIO2003-02611: 2004-2006
Project Coordinator: Lluís Ribas de Pouplana

Discovery of new antibiotics via methods of positive selection
EU Marie Curie International Reintegration Award: 2005-2006
Project Coordinator: Lluís Ribas de Pouplana

Catalan Government infrastructure grant SGR00350: 2006
Project Coordinator: Lluís Ribas de Pouplana

Support for the coordination of a European Research Network
Spanish Ministry of Science and Education grant SAF2005-24588-E: 2006
Project Coordinator: Lluís Ribas de Pouplana

Support to exceptional research groups
Catalan Government Research Department grant SGR00350:2006-2008
Project Coordinator: Lluís Ribas de Pouplana

Desarrollo de un nuevo método para la selección de antibióticos
Spanish Ministry of Science and Education grant BIO2006-01558: 2007-2009
Project Coordinator: Lluís Ribas de Pouplana

COLLABORATIONS

Selenocysteine incorporation and organelle protein synthesis in Trypanosoma
André Schneider (Department of Cell and Developmental Biology, University of Fribourg, Switzerland)

Methionine metabolism and pathogenicity in the human pathogen Mycoplasma penetrans
Rebecca Alexander (Department of Chemistry, Wake Forest University, NC, USA)

Codon ambiguity and pathogenicity of Candida albicans
Manuel Santos (Department of Biology, University of Aveiro, Portugal)

Mitochondrial protein synthesis in Drosophila melanogaster *development*
Thomas Stratmann (Department of Immunology, University of Barcelona, Spain)

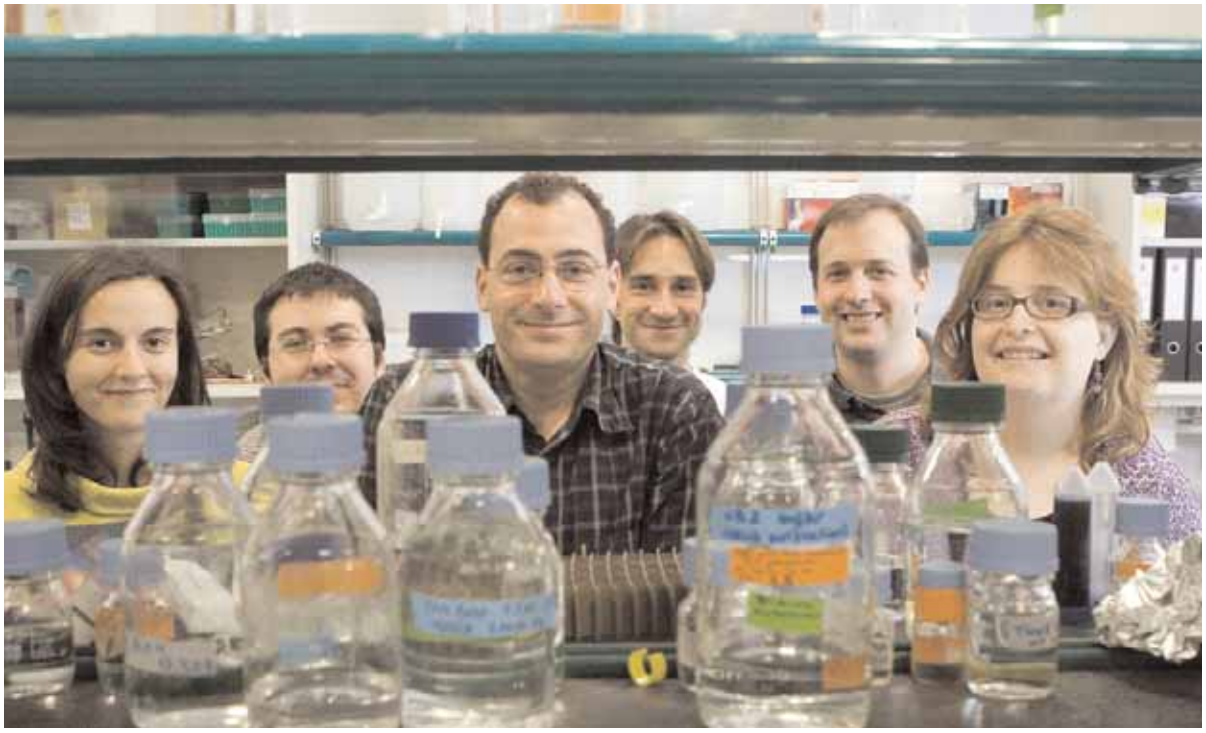
Combinatorial libraries of aminoacyl-adenylate analogues
Miriam Royo (Combinatorial Chemistry Unit, Barcelona Science Park, Spain)

Inflammatory effect of an Entamoeba histolytica *MetRS domain*
Annabel Valledor and Antonio Celada (IRB Barcelona, Spain)

Functional evolution of the glycogen metabolism
Daniel Cifuentes and Joan J Guinovart (IRB Barcelona, Spain)

Expression strategies for Plasmodium *proteins*
ERA Plantech SL, Spain

Development of positive selection screens for antibiotic discovery
Omnia Molecular SL, Spain



Lluís Ribas de Pouplana's group, March 2006.

Developmental neurobiology and regeneration

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Eduardo Soriano García

Brain development is a complex process which involves several sequential steps: regional determination, specification of neuronal cell types, control of cell migration, guidance and formation of neural connective networks, and activity-dependent synaptic plasticity. Recent research has demonstrated that these steps are exquisitely controlled by a variety of molecular and cellular mechanisms, including expression of specific transcription factors, activity of morphogens and growth factors, guidance molecules and extracellular proteins, and synaptic activity. Our research focuses on the identification of new genes involved in these processes, and the characterisation of the intracellular signalling pathways activated in growth cones in response to extracellular signals. Moreover, it is known that the adult brain does not regenerate, either after lesions or disease-associated cell-death processes. Studies on the mechanisms that govern the normal development and growth of the nervous system are essential to explain the lack of spontaneous brain repair in adult tissue and to design new regenerative approaches to repair brain lesions.

Further roles of netrins and semaphorins in neuronal guidance

We have further investigated the roles of several guidance molecules in the formation of complex brain structures, such as the cerebral cortex and the cerebellum. For instance, the way in which GABAergic interneurons in the cerebellar cortex migrate or the guidance cues steer them are not known at present. Recent data show that the development of interneurons starts at the cerebellar germinal epithelium on top of the fourth ventricle. These interneurons continue to proliferate in the postnatal cerebellar white matter and later migrate to their final position in the cerebellar cortex. We have demonstrated a chemorepulsive action of Netrin1 on postnatal cerebellar interneurons *in vitro*; we have also reported the expression pattern of Netrin1 and its receptors Dcc and Unc5 in the developing cerebellar system. Our expression results corroborate that Netrin1 is involved in the migration of GABAergic interneurons *in vivo*. Moreover, our data point to Bergmann glial fibers as possible tracks for these cells en route to the molecular layer. Finally, experiments using blocking antibodies have allowed us to conclude that Dcc, although expressed by postnatal cerebellar interneurons, is not involved in the repulsive response triggered by Netrin1 in these cells (Guijarro *et al*, 2006).

We have also studied the distribution and role of a specific variant of Semaphorin Y/6C (Sema6C) in mouse forebrain development and plasticity. Growth cone collapse of entorhinal and pyramidal neurons, as well as activation of glycogen synthase kinase-3 (GSK-3) through depletion of the inactive pool, is induced by a diffusible Sema6C1 form, suggesting a role in development. We found this isoform to be widely expressed during development, remaining in the adult and showing variations in distribution when the perforant pathway was axotomised. These changes were detected in both the hippocampal and entorhinal cortices. In axotomised animals, the ipsilateral hippocampus, but not the contralateral, hemisphere showed that Sema6C-IR had moved into the stratum lacunosum-moleculare, the medial molecular layer of the dentate gyrus (DG) and the fibers, but not the cell bodies, of the entorhinal cortex (EC). These results indicate a specific role for Sema6C variants in the generation and/or stability of circuits and synapses (Burgaya *et al*, 2006).

The tyrosine kinase Ack1/Pyk1 in brain development and plasticity

Cytosolic tyrosine kinases play a critical role both in neural development and in adult brain function and plasticity. We have isolated a cDNA that directs the expression of a 125-kD protein that can be autophosphorylated in tyrosines. This clone corresponds to the mouse homologue of Ack1 (Ack1/Pyk1) and is a non-receptor protein tyrosine kinase that comprises a tyrosine kinase core, an SH3 domain, a Cdc42-binding region, a Ralt homology region, and a proline-rich region. The highest levels of Ack1/Pyk1 expression are detected in brain, particularly in the hippocampus, neocortex, and cerebellum. Electron microscopy studies show that Ack1/Pyk1 protein is expressed both at dendritic spines and presynaptic axon terminals, indicating that this protein is involved in synaptic function. Furthermore, Ack1/Pyk1 mRNA levels are strongly upregulated by increased neural activity, which points to a role in plasticity. During development, Ack1/Pyk1 is also expressed in the proliferative ventricular zones and in postmitotic migrating and maturing neurons. These results demonstrate that this kinase is up-regulated during development and that it is expressed in proliferative areas and in migratory pathways in the developing brain. In neuronal cultures, Ack1/Pyk1 is detected in developing dendrites and axons, including dendritic tips and growth cones. Moreover, Ack1/Pyk1 colocalises with Cdc42 GTPase in neuronal cultures and co-immunoprecipitates with Cdc42s (Ureña *et al*, 2006; De la Torre *et al*, 2006). Activation of integrins by cell adhesion on fibronectin leads to strong tyrosine phosphorylation and activation of Ack. Upon cell stimulation with EGF or PDGF, Ack is tyrosine-phosphorylated and recruited to activated EGF or PDGF receptors, respectively. Moreover, tyrosine-phosphorylated Ack forms a stable complex with the adapter protein Nck via its SH2 domain (Galisteo *et al*, 2006). Taken together, our findings indicate that Ack1/Pyk1 tyrosine kinase has a functional role as an early transducer of multiple extracellular stimuli and that it may be involved in adult synaptic function and plasticity and in brain development.

The axonal growth cone: A sophisticated exploring "apparatus" designed to integrate convergent and divergent signalling pathways

During development of the nervous system, precisely ordered neuronal connections are formed in a stereotyped, stepwise process. Initially, finely orchestrated expressions of axon guidance molecules and their receptors in the projecting and the target area provide positional and directional information for ingrowing axons, which leads to a coarse connection between distinct groups of neurons. Later, activity-dependent processes, including formation and elimination of new branches, sharpen the projection, resulting in precise point-to-point connections. Throughout this process, the key apparatus of the growing axons is the neuronal growth cone. This cone could be envisaged as an exploring region at the axonal tips which integrates information from the neighbouring "milieu" to transduce signals that finally may stop or increase axonal growth. In recent years, many signalling pathways that regulate axonal navigation have been identified (*eg*, netrins, Semaphorins, Ephrins, *etc*), each bearing a full complement of receptors and associated intracellular mediators. However, how these signalling pathways, often with opposite effects, interact with each other, the hierarchy among them (if present), or how ligand/receptor complexes talk to other cell machinery, like cytoskeletal proteins and proteins regulating membrane trafficking, are not known.

Our research activities explore these issues by means of simple neuronal culture models. For instance, we have recently discovered a protein-to-protein interaction between the DCC guidance receptor and the SNARE proteins Syntaxin 1 and SNAP-25. Furthermore, these SNARE proteins are required for Netrin1/DCCinduced axonal guidance and migration, both *in vitro* and after electroporation in the spinal cord. These data point to a link between guidance receptors and the cell machinery controlling exocytosis and membrane addition (Cotrufo *et al*, in preparation).

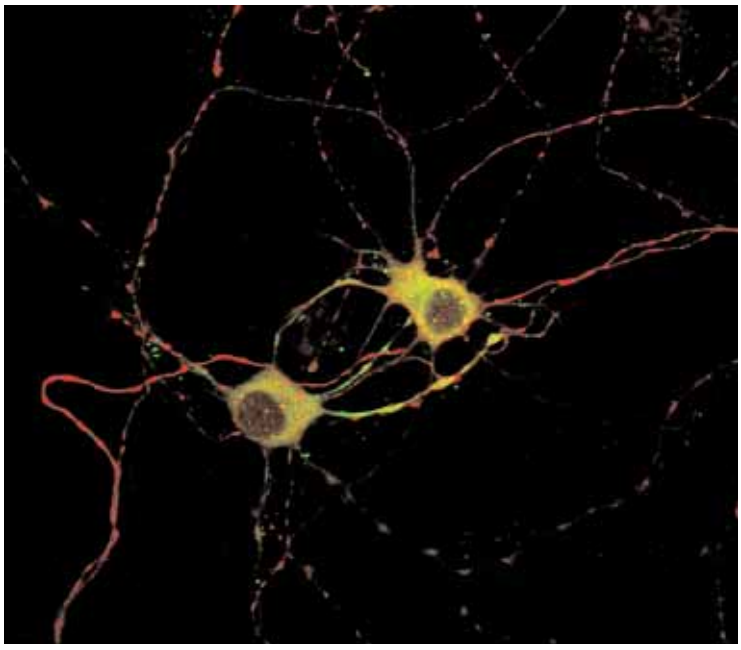

***Figure 1**. Immunocytochemical localisation of the tyrosin kinase Pyk1 (green) in cultured hippocampal neurons stained for Beta-III-Tubulin (red).*

Similarly, we explore cross-talk mechanisms between guidance molecule receptor systems. For instance, we have evidence of an interaction between the neurotrophin/trk cascade and the Netrin1/DCC and EphrinA-associated signalling pathways. We have recently shown that activation of EphrinA blocks neurotrophin-induced effects on axonal branching and synapse formation (Marler *et al*, in preparation).

Dissecting novel Reelin functions in development and neurodegenerative diseases

Reelin is a glycoprotein that is essential for the correct cytoarchitectonic organisation of the developing central nervous system (CNS). Reelin binds to very low-density lipoprotein receptor and apolipoprotein E receptor 2, thereby inducing mDab1 phosphorylation and activation of the phosphatidylinositide 3 kinase (PI3K) pathway. We have now demonstrated that Reelin activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway, which leads to the phosphorylation of Erk1/2 proteins. The inhibition of Src family kinases (SFK) blocks Reelin-dependent Erk1/2 activation. This has also been shown in neuronal cultures from mDab1-deficient mice. Although rat sarcoma viral oncogene was weakly activated upon treatment with Reelin, pharmacological inhibition of the PI3K pathway blocked Reelin-dependent Erk activation, which indicates cross-talk between the ERK and PI3K pathways. We show that blockade of the ERK pathway does not prevent the chain migration of neurons from the subventricular zone (SVZ) but does inhibit the Reelin-dependent detachment of migrating neurons. We also demonstrate that Reelin induces the transcription of the early growth response 1 transcription factor (Simó *et al*, 2006). In addition, we demonstrate a novel role of Reelin in the migration of cerebellar granule cells, which is highly dependent upon ERK activation (Simó *et al*, submitted). These findings indicate that Reelin triggers ERK signalling in an SFK/mDab1- and PI3K-dependent manner and that ERK activation is required for Reelin-dependent transcriptional activation, the detachment of forebrain neurons migrating from the SVZ, and migration of cerebellar granule cells.

The function of Reelin in the adult brain is not understood, although it has been proposed that this protein is involved in signalling pathways linked to neurodegeneration. We have analysed Reelin expression in brains and cerebrospinal fluid (CSF) from patients with Alzheimer's disease (AD) and from non-demented controls. We found a 40% increase in the Reelin protein levels in the cortex, but not in the cerebellum, of AD patients compared with controls. Similar increases were detected at the Reelin mRNA transcriptional level. This expression correlates with parallel increases in CSF but not in plasma samples. We have also studied the pattern of Reelin glycosylation by using several lectins and the anti-HNK-1 antibody. Glycosylation differed in plasma and CSF. Furthermore, the pattern of Reelin lectin binding differed between the CSF of controls and AD patients. Our results show that Reelin is up-regulated in the brain and CSF in several neurodegenerative diseases and that CSF and plasma Reelin have distinct cellular origins, thereby supporting the notion that Reelin is involved in the pathogenesis of a number of neurodegenerative diseases (Botella *et al*, 2006). To ascertain this hypothesis, we have generated a conditional transgenic mouse model that overexpresses Reelin in the forebrain. This transgenic mouse line is being crossed with several murine models of AD to ascertain whether over-activation of the Reelin pathway increases neural degeneration in these mice.

Functions of Nogo-66, MAG and CS in axonal regeneration

Damaged axons do not regenerate after axotomy in the adult mammalian CNS. This may be due to local inhibitory factors at the site of injury, such as overexpression of chondroitin sulfate (CS) proteoglycans (CSPG) and the presence of myelin-associated inhibitors. To overcome CSPG- or myelin-induced inhibition, strategies based on extrinsic and intrinsic treatments have been developed. For example, NEP1-40 is a synthetic peptide that promotes axonal regeneration by blocking Nogo-66/NgR interaction and chondroitinase ABC (ChABC), which degrades CS, thereby also promoting axon regrowth. Myelin-associated glycoprotein (MAG) also contributes to the prevention of axonal regeneration. We have studied the role of MAG, Nogo-66 and CS in the regeneration of cortical connections *in vitro*. We have shown that MAG expression is regulated in a distinct manner in the EC and the hippocampus in response to axotomy of the perforant pathway. The participation of MAG in preventing axonal regeneration was tested *in vitro*: neuraminidase treatment of axotomised entorhino-hippocampal cultures potentiates axonal regeneration (Mingorance *et al*, 2005). We have also examined whether the combination of complementary strategies facilitates axonal regeneration in slice co-cultures. Both CS cleavage with ChABC and NEP1-40 strongly facilitate the regrowth of entorhinal axons after axotomy, permitting the re-establishment of synaptic contacts with target cells. However, combined treatments do not improve the regeneration induced by ChABC alone (Mingorance *et al*, 2006). These results demonstrate that MAG, CS and Nogo-66 limit axonal regeneration in the cerebral cortex, and provide insights into the development of new assays and strategies to enhance axon regeneration in injured cortical connections.

Stem cells, neuronal precursor specification, and brain repair

The nervous system is formed by hundreds of types of neurons. The mechanisms by which the different types of neurons are generated and specified remain unclear. We have shown that, in the cerebellum, the pancreatic transcription factor *Ptf1a* is required for the specific generation of Purkinje cells and interneurons. Moreover, we have reported that granule cell progenitors in the external granule cell layer appear to be unaffected by deletion of *Ptf1a*. Cell lineage analysis in *Ptf1a*$^{Cre/Cre}$ mice was used to establish that, in the absence of *Ptf1a* expression, E12/E13-proliferating progenitors – normally fated to produce Purkinje cells and interneurons – shift to a granule cell phenotype and aberrantly migrate to the external granule layer. These findings indicate that *Ptf1a* is necessary for the specification and normal production of Purkinje cells and cerebellar interneurons, two essential GABAergic cell types of the cerebellar cortex. We have also established that *Ptf1a* is required for the suppression of the granule cell specification programme in cerebellar ventricular zone precursors (Pascual *et al*, submitted). Given the key role of this gene in Purkinje cell specification, we are now exploring whether the induced expression of this gene in neuronal stem cells of distinct origins induces their phenotypic differentiation in a Purkinje cell-like phenotype. If this is confirmed, we will have devised a method to produce Purkinje cells *in vitro*, thereby facilitating cell therapy approaches in murine models of cerebellar ataxia.

The production of neurons is a temporally restricted process that occurs during embryonic life, except in a few brain areas (the hippocampus, cerebellum, and the subventricular zone). In fact, new granule neurons are produced in the DG of rodents and humans throughout adult life. Understanding the mechanisms that control cell proliferation and neuron production in these areas is crucial to devise therapeutic strategies aimed at producing neurons from the natural "niches" that contain neural stem cells. Recent studies have also reported adult neurogenesis in the cerebral cortex of healthy animals or after brain injury. We have analysed whether the absence of the synaptic input from the main hippocampal afferents induces neuronal generation in the hippocampus outside the Dentate Gyrus (DG) and/or regulates the proliferation of DG neuroprogenitors. We have shown that the denervation of the hippocampus does not induce neurogenesis in hippocampal regions other than the DG. However, neuroprogenitor proliferation in the DG is reduced after fimbria-fornix lesions but not after entorhinal deafferentation, which supports the view that neuroprogenitor proliferation and/or differentiation in the DG are controlled from basal forebrain/septal neurons. We have also studied cell proliferation in the hippocampus of rodents and the intrinsic putative neurogenic potential of EC progenitors. We show that only the DG generates new neurons in the hippocampus. In addition, neurospheres from the EC have the capacity to differentiate into neurons and glia *in vitro* and after transplantation in the adult DG (Fontana *et al*, 2006). In a more recent study, we identified Netrin1 as a key factor controlling neurogenesis and differentiation of neural stem cells, specifically in the DG (Barallobre *et al*, in preparation) and we are currently focusing our research efforts on elucidating the cellular mechanisms that control symmetrical versus asymmetrical neural cell division.

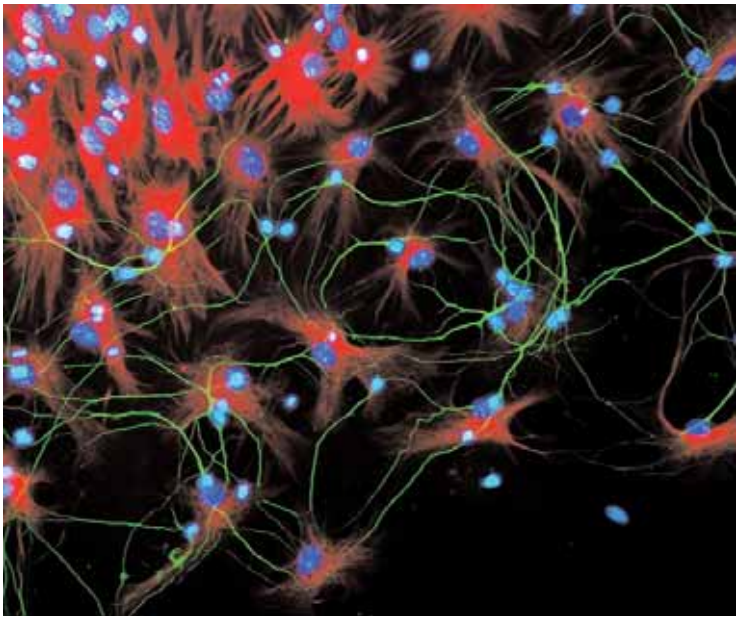

***Figure 2**. Differentiation of neurons (green) and astrocytes (red) from cultured neurospheres obtained from postnatal cerebral cortex.*

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RESEARCH NETWORKS AND GRANTS

Special Award "*Distinció per a la Promoció de la Recerca Universitària*"
Generalitat de Catalunya: 2000-2006
Project Coordinator: Eduardo Soriano García

Role of the mitochondria in neuronal death: Alex-3, a nuclear/mitochondrial protein
Ministerio de Sanidad y Consumo (ISC III): 2004-2007
Project Coordinator: Eduardo Soriano García

Developmental neurobiology and neural regeneration
"Support to established research groups"
AGAUR - Generalitat de Catalunya: 2005-2008
Project Coordinator: Eduardo Soriano García

Identification and characterisation of new genes and signalling pathways involved in cortical development
Ministerio de Educación y Ciencia: 2005-2008
Project Coordinator: Eduardo Soriano García

Potencial of the Ptf1a/p48 gene in cerebellar repair
Fundación "La Caixa": 2006-2008
Project Coordinator: Eduardo Soriano García

CIBER *"Neurodegenerative Diseases"*
Ministerio de Sanidad y Consumo: 2006-2009
Project Coordinator: Eduardo Soriano García

Characterisation of molecular mechanisms involved in the development and regeneration of hippocampal connections
Ministerio de Educación y Ciencia: 2004-2007
Project Coordinator: José A del Río

Special Award *"Distinció per a la Promoció de la Recerca Universitària"*
Generalitat de Catalunya: 2004-2008
Project Coordinator: José A del Río

Generation of cholinergic cell lines from neural progenitors: application to Alzheimer's disease models
Fundación La Caixa: 2004-2006
Project Coordinator: José A del Río

Potential of oligodendrocyte progenitors in EA disease
Fundación Mutua Madrileña Automobilistica: 2004-2007
Project Coordinator: Fernándo de Castro Soubriet

Signalling pathways in prionic diseases
Ministerio de Educación y Ciencia: 2004-2007
Project Coordinator: José A del Río and Adriano Aguzzi

Cellular and molecular bases of neurodegeneration and neurorepair
"Support to established research groups"
AGAUR - Generalitat de Catalunya: 2005-2008
Project Coordinator: José A Del Río

Role of transmembrane semaphorins in the development of epilepsia
Ministerio de Sanidad y Consumo (ISC III): 2004-2007
Project Coordinator: Ferran Burgaya i Márquez

CREB and Reelin signalling pathways in Alzheimer´s Disease
Ministerio de Sanidad y Consumo (ISC III): 2004-2007
Project Coordinator: Jesús Mariano Ureña Bares

Molecular basis of megalencephalic luekopathy: Characterisation and role of the mlc1 gene
Ministerio de Sanidad y Consumo (ISC III): 2004-2007
Project Coordinator: Albert Martínez García

Molecular mechanisms envolved in spontaneous oscillations of astrocytes: role in development and formation of circuits
Ministerio de Educación y Ciencia: 2004-2007
Project Coordinator: Fernando Aguado Tomás

COLLABORATIONS

Role of Reelin/Dab1 in prionic diseases
Adriano Aguzzi (University of Zurich, Switzerland)

Role of Netrin1 and NogoR in neural development and regeneration
Marc Tessier-Lavigne (Genentech, San Francisco, USA)

Role of Syntaxin1 and Podocalyxins in axonal guidance and brain development
Thomas Südhoff and José Rizo-Rey (Southwestern University, Dallas, USA)
Esther Stoeckl (University of Zurich, Switzerland)

Interactions between Ephrin and Trk signalling pathways in axonal navigation
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Joan X Comella (University of Lleida, Spain)

Role of CREB family transcription factors in brain development
Günther Schultz (DKMC, Heidelberg, Germany)

Role of Alex-3 in mitochondrial biology
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Functions of the novel tyrosin kinase Pyk1 in brain development
Joseph Schlessinger (Yale University, USA)

Ultrashort lasers, axonal guidance, and brain repair
Pablo Loza (ICFO, Barcelona, Spain)

Role of the pdf1 gene in cerebellar development and repair
Paco X Real (UPF/IMIM, Barcelona, Spain)

Transmembrane semaphorins and epilepsia
Javier de Felipe (Cajal Institute, Madrid, Spain)

Role of Alex-3 in Wnt/B-catenin signalling pathway
Eduard Batlle (IRB Barcelona, Spain)

The mlc1 gene in megalencephalic leukodistrophy
Manuel Palacín and Raul Estévez (IRB Barcelona, Spain)

Role of the glycogen synthase enzyme in neuronal function and degeneration
Joan J Guinovart (IRB Barcelona, Spain)

Eduardo Soriano's group, March 2006.

Structural and Computational Biology Programme

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Proteins are the main perpetrators of most cellular tasks. However, they seldom act alone and most biological processes are carried out by macromolecular assemblies and regulated through a complex network of protein-protein interactions. Thus, modern molecular and cell biology no longer focus on single macromolecules but now look into complexes, pathways or even entire organisms. The many genome-sequencing initiatives have provided a near complete list of the components present in an organism, and post-genomic projects have aimed to catalogue the relationships between them. The emerging field of systems biology is now centred mainly on unravelling these relationships. However, all these interaction maps lack molecular details: they tell us which molecules interacts with which, but not how. A full understanding of the way in which molecules interact can be attained only from high resolution three-dimensional (3D) structures, since these provide crucial atomic details about binding. These details allow a more rational design of experiments to disrupt an interaction and therefore to perturb any system in which the interaction is involved. Our main scientific interests are in the field of structural bioinformatics, in particular, the use of protein sequences and high-resolution 3D structures to reveal the molecular bases of how macromolecular complexes and cell networks operate.

Target selection for complex structural genomics
Large-scale interaction discovery experiments are revealing the thousands of interactions and protein complexes responsible for most cellular functions, although they usually lack the molecular details that explain how the interactions occur. High-resolution 3D structures provide atomic information about the interaction interfaces. However, because of the difficulty of the experiments, the number of complexes of known structure is still limited. The launch of structural genomics initiatives focused on protein interactions and complexes could quickly fill the interaction space with structural details, thereby offering a new perspective on how cell networks operate at atomic level. Clear target selection strategies that rationally identify the key interactions and complexes that should be tackled first are fundamental to maximise return, minimise costs and prevent experimental difficulties. A complete interaction space filled with atomic-level details for each interaction, complex, signalling cascade and metabolic pathway would provide the perfect framework for future developments in systems biology.

We recently became a partner of the first-ever *complex* structural genomics initiative, named 3D repertoire, which seeks tos solve the structures of all amenable protein complexes in yeast at the highest resolution possible. The project involves 20 institutions across Europe and a multidisciplinary team of over 100 scientists in the fields of X-ray crystallography, NMR, electron microscopy and bioinformatics. We have been entrusted with the task of devising and implementing a strategy for selecting a set of complexes that stand a good chance of undergoing successful expression, purification and crystallisation. For this purpose, we have developed an automated procedure that combines several types of biological data (*eg*, socio-affinities, sub-cellular localisation, protein abundance, yeast two-hybrid experiments, *etc*) in a quantitative manner and scores each complex/sub-complex in yeast on the basis of its chances of success under standard expression and purification conditions. The system allows certain flexibility and we have also developed a web interface to permit users choose their own ranking criteria (http://gatealoy.pcb.ub.es/targetselection). The final result is a dynamic system to produce ranked lists of protein complexes/sub-complexes. The target selection strategy, the web-tool and the results have been disseminated among the partners of 3D repertoire and will shortly be available to the whole scientific community. We are currently expanding the target selection system to attach a confidence value to each complex/sub-complex and to extend it to other model organisms such as the fly, worm, mouse or human.

Contextual specificity in peptide-mediated protein interactions

Protein interactions are key to virtually every major process in a cell. While large protein-protein interfaces are typical in tightly associated macromolecular complexes (*eg*, RNA polymerase II), many transient interactions are mediated by a globular domain in one protein that recognises a small linear peptide in another (*eg*, SH3 domain recognising a proline-rich motif). However, although it has been shown that these motifs are enough to ensure binding, they are usually too short (4 to 11 residues) to achieve the high specificity observed in these interactions. It is thus a more general context outside the small linear peptide (*ie*, other interacting residues, subcellular localisation, expression patterns, *etc*) that will ultimately determine the interaction between two given proteins. (See Figure 1.)

In the lab, we have used high-resolution 3D structures of interacting protein pairs to explore the contribution of the binding motif and surrounding residues (the context) in all known domain-ligand interaction types. We have found that, on average, contextual contacts account for roughly 30% of the binding energy. We have also seen that the central motif itself is fairly unspecific as it has the capacity to bind to many homologous domains. Therefore it is the context, to a large extent, that is responsible for the high affinity shown by this type of interaction, either by improving the binding energy with the native partner or preventing non-native interactions. We are currently exploring the bearings that our findings have in systems biology, since they might provide a rationale for several compensatory effects observed in knock-out networks and phenotypic profiles, and in synthetic biology, where specificity information is instrumental in the construction of artificial cellular circuits. (See Figure 2.)

Pushing structural details into protein interaction networks

Although there is a growing gap between the number of interactions detected and those for which the 3D structure is known, it is equally true that, since the mid-nineties, crystallographers have been solving structures at a rate of over one thousand interactions every year. Thus, the Protein Data Bank currently contains many thousands of interactions for which structural data are available, which implies that it is increasingly possible to model structures for protein interactions on the basis of those observed previously. Like most modelling efforts, accuracy depends greatly on the degree of sequence identity between the target and the template onto which it is modelled. When modelling an interaction, the choice of the template is all the more crucial because the use of the wrong template may lead to protein interactions through the incorrect interface. This is roughly analogous to modelling a single protein on another that has a different fold. Encouragingly, however, when sequence similarity is high (for example, >25-30% identity) proteins are likely to interact in the same way, although exceptions are possible. There are instances where interactions are structurally similar despite there being no sequence similarity; the trick is to find them. The last five years have seen the emergence of a new class of techniques that use protein-protein complexes for which coordinate data are available to model interactions between their homo-

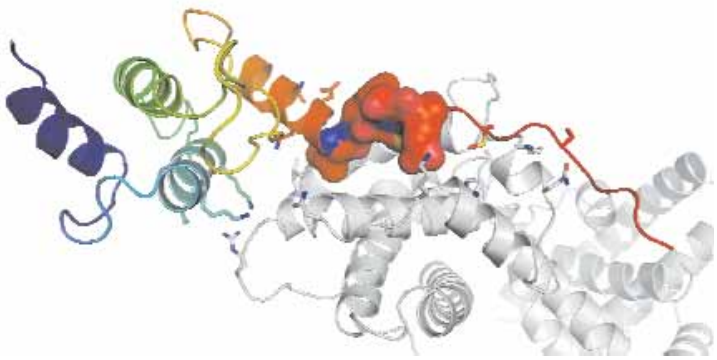

***Figure 1**. Example of contextual specificity. Interaction between the retinoblastoma protein (grey) and a viral peptide (rainbow). The consensus motif ([LI].C.[DE]) responsible for the binding is shown in a surface representation. Selected contextual interactions are highlighted in a stick model, some of which are at a considerable distance from the motif.*

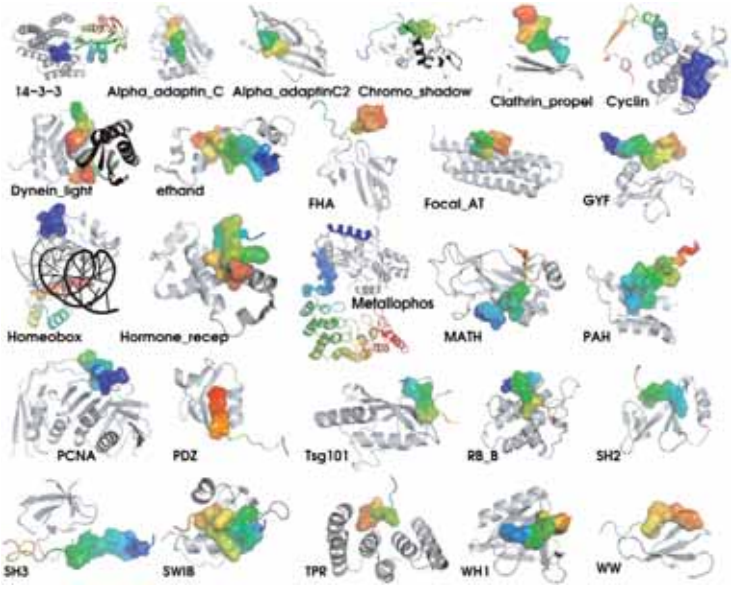

***Figure 2**. Transient domain-peptide interactions of known 3D structure. Collection of representative structures of interaction domains (grey) bound to their partner proteins (rainbow) containing the binding linear motifs (surface).*

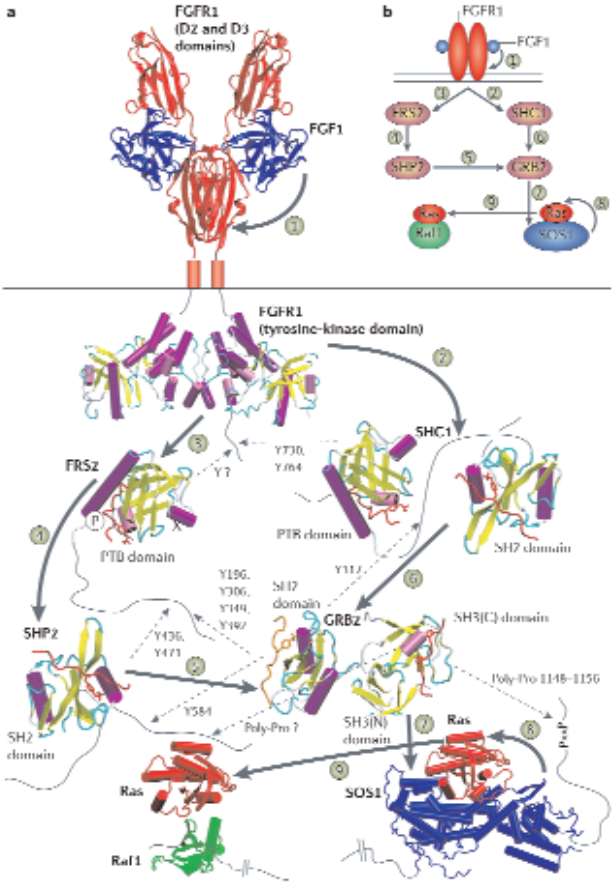

***Figure 3**. The fibroblast growth factor signalling pathway. Structural details inferred for the fibroblast growth factor (FGF) pathway (a) as a means to complement its classical blob-representation shown in the text books (b). X-ray, NMR or modelled structures are shown in diagram format (α-helices are shown as cylinders and β-strands are depicted as arrows). Structures of complexes of two large proteins (for example, FGF/FGFR) are coloured according to chain (that is, each separate polypeptide is a different colour); structures involved in domain-peptide or phosphorylation interactions are coloured according to the secondary structure of the domain (helices are magenta and β-strands yellow) and the interacting peptides are red, or orange or are shown schematically. Black arrows denote the activation events highlighted in the schematic diagram, dashed arrows show interactions between domains in one protein and particular regions on another; the labels on these arrows indicate the residues where the domain binds (when known). (Aloy and Russell, 2006.)*

logues. However, these approaches are far from perfect, and they suffer when the interactions involve conformational changes at the interface, or when the modelled interfaces contain insertions or deletions, with respect to the template, that are not accurately modelled. Moreover, they are usually unable to sort out the correct specificity between members of two interacting families and the results given often do not have any biophysical meaning (*ie*, there is no correlation between computationally derived scores and, for instance, dissociation constants). We are now working on a novel approach to tackle the specificity problem and to predict binding energies for domain-domain interactions by means of empirical pair potentials. We are also developing an automated strategy to use these potentials to predict new protein-protein interactions on a genome scale. Being structure-based, the resulting networks will not only tell us which molecules interacts with which but will also give quasi-atomic information as to how these interactions occur. This level of detail will permit a much more rational design of experiments to disrupt an interaction and therefore to perturb the global behaviour of the network.

Structural systems biology: explaining macroscopic effects at molecular level

One of the major goals of the emerging field of systems bology is to explain macroscopic effects at molecular level. This is to build computational models to simulate the behaviour of complex systems, such as limb development in mice, or the cellular circuits responsible for certain phenotypes in yeast or *Arabidopsis*. We aim to combine the knowledge we have acquired from the above projects (*ie*, the molecular determinants of specificity in the different types of protein-protein interactions, structure-based interaction networks, *etc*) with other types of data available (*ie*, protein expression, interaction kinetics, *etc*) in order to propose potential new pathways that explain the observed phenotypic profiles observed in model organisms. One of the first steps that we have taken towards this end is to push quasi-atomic details into known signalling pathways, wherever possible, to convert the classical, and not very informative, blob-diagrams into something more structurally meaningful that can provide much clearer insights into the events that occur. (See Figure 3.)

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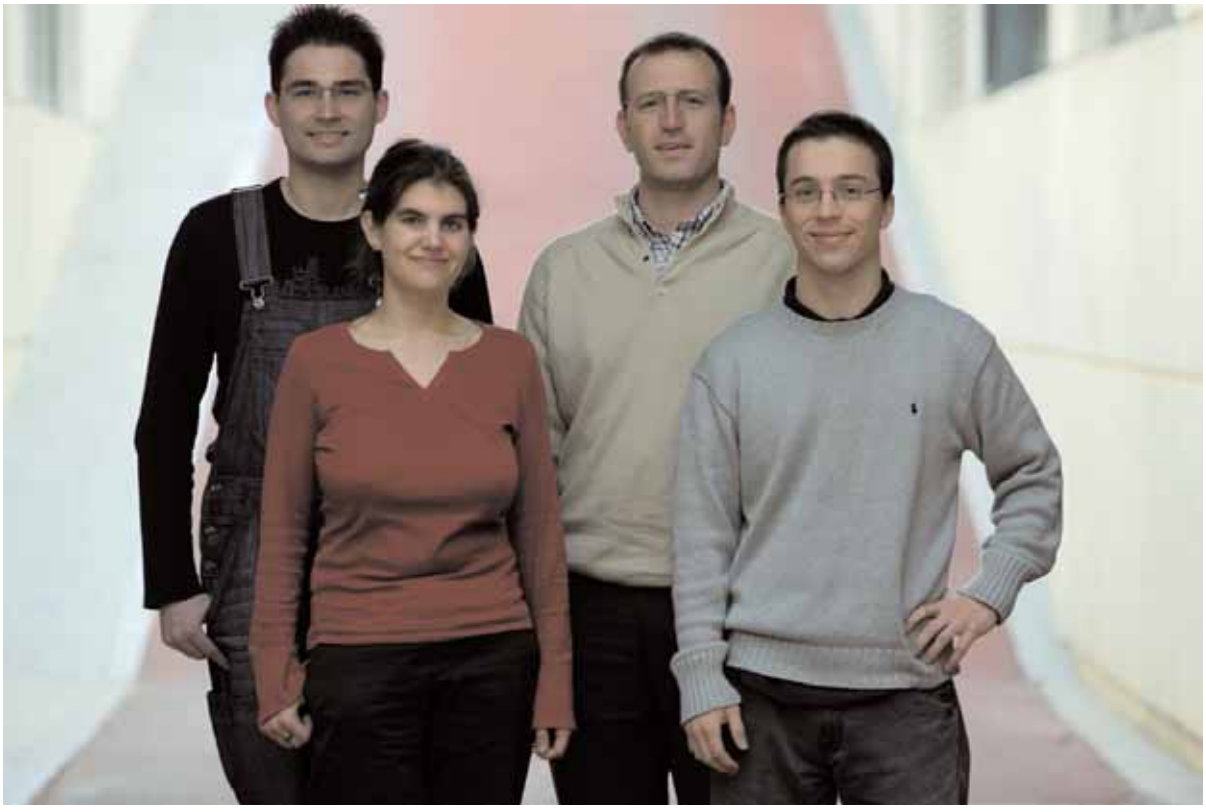
RESEARCH NETWORKS AND GRANTS

A multidisciplinary approach to determine the structures of protein complexes in a model organism
European Integrated Project, European Commission: 2005
Project Coordinator: Luis Serrano



Patrick Aloy's group, March 2006.

Structural biology of proteins, nucleic acids and their complexes

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Miquel Coll

Our research focuses on the 3D structure of proteins, nucleic acids and their complexes with the aim to further our understanding of several essential mechanisms in the cell. We use a number of molecular biology and structural biology techniques, with a focus on X-ray diffraction crystallography. We examine systems related to horizontal gene transfer which involve DNA translocation across the cell membrane. In addition, we address the regulatory mechanisms of gene expression and the control mechanisms of DNA replication. We also study unique DNA structures, like DNA junctions, and novel drugs that target DNA.

Horizontal gene transfer

Conjugation is the main route for horizontal gene transfer in bacteria and is responsible for the spread of antibiotic resistance. During conjugation, plasmid DNA is processed and then transported across cell membranes between cells (Rüth and Coll, 2006). DNA processing involves a key protein called relaxase, which recognises an extruded DNA hairpin at the *Origin of Transfer*, nicks the DNA and separates one of the strands, which will be subsequently transferred. After our studies leading to the resolution of the first structure of a relaxase-DNA complex, we have deepened our analysis of the molecular mechanism involved in the relaxase activity by solving a protein-DNA complex (Figure 1) that includes the scissile phosphate. This study shows that a metal ion is required for phosphate polarisation or reaction intermediate stabilisation to facilitate the nucleophilic attack of the catalytic tyrosine of the relaxase (Boer *et al*, 2006).

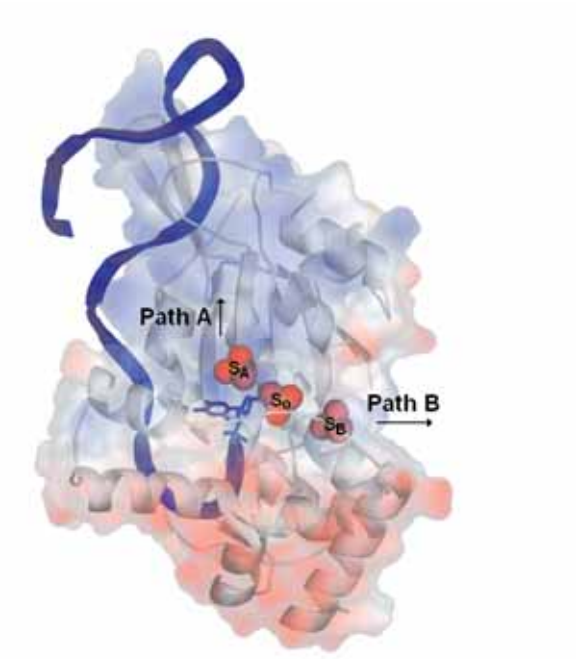

Figure 1. *TrwC relaxase nicks the DNA after binding to an extruded DNA hairpin at the plasmid Origin of Transfer during bacterial conjugation (Boer* et al, *2006).*

Transcription regulation

We study several transcription factors and their complexes with other proteins and DNA promoter regions. In the bacteriophage ϕ29, we have examined the transcriptional regulator p4, which functions as a switch between early and late gene expression during the infection cycle. By solving the structure of the p4 dimer bound to a 41 bp-long promoter DNA sequence, we have revealed how this is achieved (Badia *et al*, 2006). A delicate interaction, involving only one base-specific recognition contact at each tip of the elongated p4 dimer, is mediated by a novel DNA binding motif that we have termed *N-hook* (Figure 2).

In another study we have addressed the *E. coli* PhoB transcriptional activator, a response regulator of the two-component signal transduction system that con-

trols the expression of more than 40 genes related to phosphate assimilation. Constitutively active mutants of the protein have been structurally characterised in order to examine the mechanism leading to PhoB activation (Solà *et al*, 2006; Arribas-Bosacoma *et al*, 2006). Finally, a ternary complex including the transcription factor, the DNA binding sequence and the C-term, a subunit of the RNA polymerase, has been solved, showing how the polymerase is recruited to the promoter region (Blanco *et al*, in preparation).

DNA structure and drug-DNA interactions

We are currently analysing unique DNA structures, such as four-way and three-way junctions related to DNA recombination and other processes. A novel cytotoxic drug consisting of a supramolecular helicate has been shown to bind to a three-way junction DNA with a perfect fit in the central trigonal cavity of the junction (Oleksi *et al*, 2006). Electrostatic and stacking interactions stabilise the interaction in a binding mode that has not been described previously (Figure 3).

Structural genomics

Considerable research effort is devoted to setting up medium/high throughput technologies for the expression and crystallisation of proteins and complexes (Fogg *et al*, 2006). In relation to these activities, our group participates in several National and European Structural Genomics consortia:
VIZIER (www.vizier-europe.org),
3D-REPERTOIRE (www.3drepertoire.org),
GENES (ub.cbm.uam.es/genes)
and SPINE 2-Complexes (www.spine2.eu).

***Figure 2**. Bacteriophage Phi29 transcriptional regulator p4 acts as a molecular switch controlling the phage infection cycle by turning off early genes and turning on late genes (Badia* et al, *2006).*

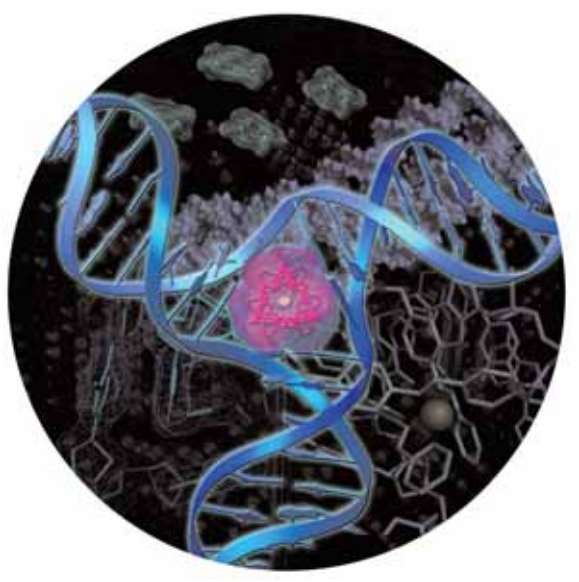

***Figure 3**. A novel cytotoxic drug, a supramolecular helicate, recognises a three-way junction DNA (Oleksi* et al, *2006).*

PUBLICATIONS

Arribas-Bosacoma R, Kim S-K, Ferrer-Orta C, Blanco AG, Pereira PJB, Gomis-Rüth X, Wanner BL, Coll M and Solà M (2006) The X-ray crystal structures of two constitutively active mutants of the *E. coli* PhoB receiver domain give insights into activation. J Mol Biol, 366:626-641

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Boer R, Russi S, Guasch A, Lucas M, Blanco AG, Pérez-Luque R, Coll M and de la Cruz F (2006) Unveiling the molecular mechanism of a conjugative relaxase: The structure of TrwC complexed with a 27-mer DNA comprising the recognition hairpin and the cleavage site. J Mol Biol, 358:857-869

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Gomis-Rüth FX and Coll M (2006) Cut and move: DNA processing in bacterial conjugation. Curr Opin Struct Biol, 16:744-752

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Solà M, Drew DL, Blanco AG, Gomis-Rüth FX and Coll M (2006) The co-factor-induced pre-active conformation in PhoB. Acta Crystallogr D Biol Crystallogr, 62:1046-1057

RESEARCH NETWORKS AND GRANTS

Estructura de proteínas de unión al DNA
Proyecto Plan Nacional de I+D+I BIO2002-03964
Ministerio de Ciencia y Tecnología: 2002-2005
Principal Investigator: Miquel Coll

Structural effects arising from major groove DNA recognition by metallo-supramolecular cylinders (MARCY)
Research Training Networks RTN2-2001-00230
European Union: 2002-2006
Principal Investigator: Miquel Coll

Genómica estructural: aplicación a proteínas y complejos proteicos relacionados con el cáncer
Proyecto Integrado GEN2003-20642. Acción estrategica de genómica y proteómica
Ministerio de Ciencia y Tecnología: 2004-2007
Principal Investigator and Coordinator: Miquel Coll

Comparative structural genomics of viralenzymes involved in replication (VIZIER)
Integrated Project VI PM LSHG-CT-2004-511960
European Union: 2004-2008
Principal Investigator: Miquel Coll

A multidisciplinary approach to determine the structures of protein complexes in a model organism (3D-REPERTOIRE)
Integrated Project VI PM LSHG-CT-2005-512028
European Union: 2005-2009
Principal Investigator: Miquel Coll

Estructura de proteínas y complejos de unión al DNA
Proyecto Plan Nacional de I+D+I BFU2005-06758/BMC
Ministerio de Educación y Ciencia: 2005-2008
Principal Investigator: Miquel Coll

Grupo de cristalografía de proteínas
Ayudas a Grupos de Investigación Consolidados 2005SGR-00280
CIRIT, Generalitat de Catalunya: 2005-2008
Principal Investigator: Miquel Coll

Epigenetic chromatin regulation proteins as targets in anticancer strategy
Proyecto Fundació La Marató de TV3 052810
Fundació La Marató de TV3: 2005-2008
Principal Investigator: Miquel Coll

Ayuda complementaria al proyecto: Genómica estructural comparativa para enzimas víricos
Acción complementaria de investigación BFU2005-24122-E
Ministerio de Educación y Ciencia: 2006-2010
Principal Investigator: Miquel Coll

Ayuda complementaria al proyecto: Una aproximación multidisciplinaria para determinar las estructuras de los complejos proteicos en un organismo modelo
Acción complementaria de investigación BFU2005-24123-E
Ministerio de Educación y Ciencia: 2006-2010
Principal Investigator: Miquel Coll

From receptor to gene: structures of complexes from signalling pathways linking, immunology, neurobiology and cancer (Spine II Complexes)
Integrated Project VI PM LSHG-6 Proposal 031220
European Union: 2006-2010
Principal Investigator: Miquel Coll

Consorcio para el descubrimiento y desarrollo de nuevos fármacos (GENIUS PHARMA)
Proyecto CENIT
CDTI (Ministerio de Industria, Turismo y Comercio): 2006-2009
Principal Investigator: Miquel Coll

CENTROSOMA 3D: Hacia la comprensión estructural y funcional del centrosoma
Proyecto CONSOLIDER- INGENIO 2010
Minsterio de Educacion y Ciencia: 2006-2011
Principal Investigator: Miquel Coll

COLLABORATIONS

Plasmid replication
Manuel Espinosa and Gloria del Solar (Centro de Investigaciones Biológicas, CSIC, Spain)

Bacterial conjugation
Fernando de la Cruz (Universidad de Cantabria, Spain)

DNA packaging
José L Carrascosa and José María Valpuesta (Centro Nacional de Biotecnología, CSIC, Spain)

Transcription regulation
Margarita Salas (Centro de Biología Molecular, CSIC, Spain)

Transcription regulation
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Transcription regulation
Juan Aguilar (Universitat de Barcelona, Spain)

Transcription regulation
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HTP protein expression
Darren J Hart (EMBL Heidelberg, Germany)

DNA-drugs
Mike Hannon (University of Birmingham, UK)

Tubulin folding co-factors
Juan Carlos Zabala (Universidad de Cantabria, Spain)

Prolyl oligopeptidases
Ernest Giralt (IRB Barcelona, Spain)

Growth factors
Jordi Casanovas (IRB Barcelona, Spain)

Chromatin-modifying proteins
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CENIT project Genius Pharma
CrystaX Pharmaceuticals, SL (Barcelona, Spain)

Miquel Coll's group, March 2006.

Structural biology of macromolecular aggregates

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During the last fifteen years our research has focused on the structure-function relationships of a diversity of proteins and macromolecular complexes directly involved in pathological processes. The systems studied, with over a hundred peer-reviewed publications, range from a number of small protein kinase domains to several intact RNA viruses and in complex with their cellular receptors. To perform our activities, we use mainly X-ray crystallography, often complemented with other techniques including, in particular, mass spectrometry and high resolution cryo-electron microscopy.

Our general goal is first to determine the three-dimensional organisation of a number of molecular systems, and second, to use these data as a framework to examine the biochemical and biological processes in which these molecular systems participate. The development of methodologies required by some of these studies has also been an objective in itself.

At present our main lines of research are: Enzymes related with Oxidative Stress (a) Mono-functional Catalases, b) Bifunctional Catalases-Peroxidases, c) Peroxidases (in particular mammalian peroxidases) and d) Oxygenases; Membrane bound proteins a) Amino-acid transporters and b) Virulence factors (in particular in Mycoplasmas); Energetics of protein conformations

PUBLICATIONS

Calisto BM, Pich OQ, Piñol J, Fita I, Querol E and Carpena X (2005) Crystal structure of a putative type I restriction-modificatrion S subunit from *Mycoplasma genitalium*. J Mol Biol, 351:749-762

Carpena X, Wiseman B, Deemagarn T, Herguedas B, Loewen B and Fita I (2006) pH dependence of the structure and activity of catalase-peroxidase KatG from *Burkholderia pseudomallei*. Biochemistry, 45:5171-5179

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Chelikani P, Carpena X, Perez-Luque R, Donald LJ, Duckworth HW, Switala J, Fita I and Loewen PC (2005) Characterization of a large subunit catalase truncated by proteolytic cleavage. Biochemistry, 44:5597-5605

Deemagarn T, Carpena X, Singh R, Wiseman B, Fita I and Loewen PC (2005) Structural characterisation of the Ser324Thr variant of the catalase-peroxidase (KatG) from *Burkholderia pseudomallei*.J Mol Biol, 345:21-28

Deemagarn T, Wiseman B, Carpena X, Ivancich A, Fita I and Loewen P (2006) Two alternative substrate paths function in catalase-peroxidase KatG from *Burkholderia pseudomallei*. Proteins: Structure, Function and Bioinformatics, 66:219-228

Horcajada C, Guinovart J, Fita I and Ferrer JC (2006) Crystal structure of an archaeal glycogen synthase: Insights intooligomerisation and substrate binding of eukaryotic glycogen synthases. J Biol Chem, 281:2923-2931

Querol J, Fita I and Verdaguer N (2005) X-ray crystallography of rhinovirus-receptor complexes. Crystallography Reviews, 11:73-81

Querol J, Perez-Luque R, Fita I, Lopez C, Gastón JR, Carrascosa JL and Verdaguer N (2005) Preliminary analysis of two and three dimensional crystals of vault ribonucleoprotein particles. J Struct Biol, 151:111-115

Ramón-Maiques S, Fernandez-Murga ML, Gil-Ortiz F, Vagin A, Fita I and Rubio V (2006) Structural bases of feedback control of arginine biosynthesis, revealed by the structures of two hexameric N-acetylglutamate kinases, from *Thermotoga maritima* and *Pseudomonas aeruginosa*. J Mol Biol, 356:695-713

Rosell A, Valencia E, Borras, E, Ochoa WF, Fita I, Pares X and Farres J (2005) Structural plasticity in alcohol dehydrogenase: How an NADP(H)-dependent enzyme becomes specific for NAD(H). Enzymology and Molecular Biology of Carbonyl Metabolism, 12:181-189

Rovira C, Alfonso-Prieto M, Biarnés X, Carpena X, Fita I and Loewen PC (2005) A first principles study of the binding of formic acid in catalase complementing high resolution X-ray structures. Chemical Physics. 323: 129-137

Trapani S, Abergel C, Gutsche I, Horcajada C, Fita I and J. Navaza (2006) Combining experimental data for structure determination of flexible multimeric macromolecules by molecular replacement. Acta Cryst. D62:467-475

RESEARCH NETWORKS AND GRANTS

Estructura de enzimas implicados procesos patológico
BIO2002-04419-C02-01
Ministerio de Ciencia y Tecnología: 2002-2005

Estructura y función de hemo-enzimas
BIO2005-08686-C02-01
Ministerio de Educación y Ciencia: 2006-2008

Ignasi Fita's group, March 2006.

Biomolecular NMR spectroscopy

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Begoña Morales
Ximena Ramirez

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Lidia Ruiz

Visitor
Ayelen Pagani (Argentina)

Maria J Macias

The main focus of our research is the study of protein-protein interactions at an atomic level, with the objective to provide 3D structures that contribute to a better description of biological processes. We apply multidimensional nuclear magnetic resonance (NMR) spectroscopy in conjunction with other biophysical and biomolecular techniques in order to characterise the properties of the proteins and ligands under study. Our activities are therefore centered not only on the structural determination of proteins and complexes but also on deciphering the mechanisms that contribute to the definition and maintenance of a given fold, or to the control of a given interaction. Our research involves the efficient coordination of the following: a) A wet lab for protein cloning, expression and purification aimed at obtaining labelled protein samples required for NMR experiments. We have recently incorporated small scale solid-phase peptide synthesis, which allows the preparation of peptides required for ligand binding studies at a low cost and in a short time. b) Set-up of NMR experiments at the spectrometer. c) Internal computational facility, which allows the processing of NMR data, and the assignation, calculation and analysis of structures. We have also installed our own computational grid in the lab to process parallel jobs. d) Development of software packages, which cover data processing and assignment to structure calculation, in order to facilitate the processes of structural determination by NMR. In addition, several programmes and protocols developed in our lab are available free of charge and can be either directly downloaded from our group web page, or sent upon request.

The pleckstrin DEP domain structure
DEP domains are divergent in sequence. Using multiple sequence alignments and phylogenetic tree reconstructions, we found at least six subfamilies of DEP domains. We selected Pleckstrin, the major substrate of PKC in platelets, for further structural research. We found that the Pleck-DEP fold contains an additional short helix α4 inserted in the β4-β5 loop with respect to other DEP structures. This helix exhibits increased backbone mobility, as shown by NMR relaxation measurements, and may be involved in protein-protein interactions. (Civera *et al*, 2005.)

Unraveling the protein-protein interaction scenario during splicing: implication of FF domains
To clarify the functional roles of other domains present in the Prp40 splicing factor, we studied the structure and ligand preferences of its fist FF domain. Our results revealed that not all FF domains in Prp40 are functionally equivalent. We proposed that FF domains have at least two distinct interaction surfaces, which have evolved to recognise distinct binding motifs. This is an ongoing project since we are now examining the role of other divergent FF domains present in Prp40 and in CA150. (Gasch *et al*, 2006.)

Phosphorylation of the Itch WW domain does not affect its 3D structure
Recent evidence shows that the ubiquitin ligase Itch can be efficiently phosphorylated *in vivo* at several positions. Since domain phosphorylation is quite unusual (it normally occurs at flexible linkers that connect domains), we sought to clarify whether the Itch WW domain maintains its fold when phosphorylated. We expressed the wild-type domain and Dr. Miriam Royo's group at the PCB synthesised the phosphorylated variants. Our structural work supports the view that in the three cases examined (wild-type and phosphorylated mutants) the WW structure is maintained, thereby opening up the possibility of regulating ligand binding upon domain phosphorylation. (Shaw *et al*, 2005.)

Regulation of ligand binding by serine/threonine phosphorylation of WW domains
Using NMR we have studied the role of phosphorylation as a regulatory mechanism for the interaction between the E3 ubiquitin-ligase ItchWW3 domain and two PPxY motifs of one of its targets, the Epstein-Barr virus latent membrane protein 2A. While ligand phosphorylation only diminishes binding, domain phosphorylation at T30 abrogates it. We show that two Itch WW domains can be phosphorylated at this

position using CK2 and PKA kinases and/or with stimulated T-lymphocyte lysates. To better describe the regulation process, we solved the structures of the ItchWW3-PPxY complex and the phosphoT30-ItchWW3 variant. The peptide binds the domain using both XP and tyrosine grooves. A hydrogen bond from T30 towards the ligand is also observed. This hydrogen bond formation is precluded in the variant, which explains the inhibition observed upon phosphorylation. Our results indicate that phosphorylation at position 30 in WW domains could be a mechanism to inhibit target recognition *in vivo*. (Morales et al, 2007.)

New ligand binding modes in WW domains

We have recently found that the second domain of the transcription factor CA150 binds a new motif, which we named PPLIPP, in at least two distinct modes, both of which use a newly identified cavity known as the XP2 groove. In collaboration with Dr. Carles Suñe (CSIC-Madrid), we are currently studying the interactions of this domain with other ligands containing variable PPxxxPP motifs (manuscript in preparation).

Software development: automatic assignment of NMR data

The main bottleneck of the NMR process still lies at the data-analysis step, called resonance assignment. We have developed a software package that combines peak-picking and assignment processes, thereby improving performance in both tasks and drastically reducing user intervention and assigning not only protein backbone but also carbon side-chains. At present, we are working on the automated assignment of protons. (Martin-Malpartida P and Macias MJ; in preparation).

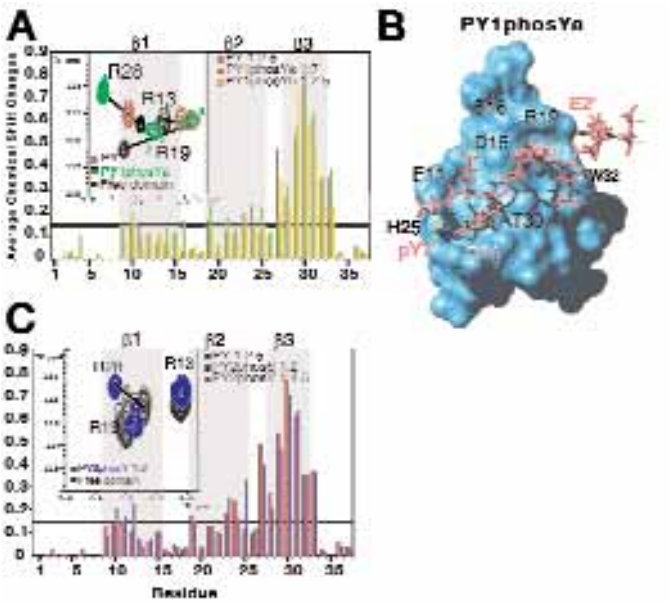

Figure 1. *Titration experiments for the phosphorylated domain variants. A. Superimposition of pT30 (phosphorylated T30) TOCSY spectrum in the free (black), and after the addition of 7 times excess of PY peptide (magenta). Some residues located in the domain's binding site are labelled for clarity. Even in the presence of high excess of peptide, no changes in the domain resonances are observed. B. Identical region of the TOCSY spectrum displayed in A but for the pS16 variant. In this case, the addition of a 3:1 peptide excess induces changes very similar to the ones observed for the wild type. Again, the reference is shown in black while the complex is displayed in magenta. Some residues in the binding site are also labelled. The inset displays the alpha and amide chemical shift differences calculated for the 3:1 complex with respect to the free pS16 chemical shifts. C. Surface and cartoon representation of the pT30 structure calculated from the NOESY experiment of ItchWW3 pT30 variant showing that the phosphorylation of T30 partially reduces the XP binding cavity, used to interact with the ligand prolines.*

PUBLICATIONS

Civera C, Simon B, Stier G, Sattler M and Macias MJ (2005) Structure and dynamics of the human pleckstrin DEP domain: distinct molecular features of a novel DEP domain subfamily. Proteins, 58:354-366

Gasch A, Wiesner S, Martin-Malpartida P, Ramirez-Espain X, Ruiz L and Macias MJ (2006) The structure of Prp40 FF1 domain and its interaction with the crn-TPR1 motif of Clf1 gives a new insight into the binding mode of FF domains. J Biol Chem, 281:356-364

Morales B, Ramirez-Espain X, Shaw AZ, Martin-Malpartida P, Yraola F, Sanchez-Tillo E, Farrera C, Celada A, Royo M, Macias MJ (2007) NMR structural studies of the ItchWW3 domain reveal that phosphorylation at T30 inhibits the interaction with PPxY-containing ligands. Structure, 15:473-483

Shaw AZ, Martin-Malpartida P, Morales B, Yraola F, Royo M and Macias MJ (2005) Phosphorylation of either Ser16 or Thr30 does not disrupt the structure of the Itch E3 ubiquitin ligase third WW domain. Proteins, 60:558-560

RESEARCH NETWORKS AND GRANTS

Protein dynamics, ligand recognition and structural characterisation of complexes applying Nuclear Magnetic Resonance
1.GEN2003-20642-C09-04/NAC: 2004-2007
Project Coordinator: Ernest Giralt

Structure determination of FF domains and characterisation of their interactions by multidimensional Nuclear Magnetic Resonance
2.BFU2005-06276: 2005-2008
Project Coordinator: Maria J Macias

COLLABORATIONS

Studies on the interactions between Ca150 WW and FF domains and their ligand counterparts
Carles Suñe (Parque Tecnológico de Ciencias de la Salud, Granada, Spain)

Peptide synthesis
Miriam Royo (Parc Científic de Barcelona, Universitat de Barcelona, Spain)

Studies on ubiquitin ligases
Antonio Celada (IRB Barcelona, Spain)

Maria Macias' group, March 2006.

Molecular modelling and bioinformatics unit

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Postdoctoral Fellows
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Tim Meyer
Agnés Noy
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David Piedra
Josep Ramón Goñi
Albert Solernou
David Talavera

Research Assistants
Iván Párraga
Margarita Pedro
Iván Sánchez

Visitor
Jiali Gao (USA)


Modesto Orozco

Our long term objective is to understand the behaviour of living organisms by means of theoretical models, the roots of which are anchored in the basic principles of physics and chemistry. With this aim, we work with a number of methodologies, from mining of biological databases to classical dynamics and quantum chemistry calculations. This wide range of methodologies has allowed us to explore topics as diverse as enzyme reactivity and genome analysis.

Small model systems
Our group has a long trajectory in the study of small model systems of biological importance (nucleobase complexes, drugs, isolated complexes of amino acids, stacked or hydrogen-bonded complexes). The analysis of these simple systems can contribute to our understanding of the behaviour of more complex biological molecules. Almost a decade ago, we realised that these studies were simple in the gas phase but very difficult in aqueous solution, thereby hampering the real application of the information obtained to the biological scenario. This consideration led us to the development of methods to describe solvent, some of which are regarded as the "state of the art" in the field (for a review see Orozco and Luque, 2000).

During 2006 we have continued to develop and improve methods to study condensed phases and to study the application of these and other methods to describe model systems of biological interest. Thus, in collaboration with Tomasi's group, we have improved continuum methodology by introducing dispersion contributions in an accurate way (Curutchet *et al*, 2006), a methodology successfully applied to complex chemical processes (Soteras *et al*, 2006; Blas *et al*, 2006). We have also made progress in transferring the methodology developed towards the macromolecular scenario (nucleic acids: Muñoz-Muriedas *et al*, 2006 or proteins: Talavera *et al*, 2006). Regarding application, we should highlight the first complete *ab initio* atlas of nucleobases-nucleobase stacking (Sponer *et al*, 2006) done by our group in collaboration with the team led by Hobza. We have also worked intensively on the analysis of expanded oligonucleotides, non-natural derivatives of DNA bases that can be incorporated in DNA, thereby changing its properties. We have explored the behaviour of these molecules and the differences they are expected to introduce in the structure/reactivity of DNA (Huertas *et al*, 2006a, 2006b; Fuentes *et al*, 2006). These model calculations have made it possible to conduct current work on the simulation of large fibers of expanded nucleobases (Huertas *et al*, unpublished).

Structural studies on proteins
Our group has extensive experience in the study of individual proteins of biological importance. Our studies are typically in collaboration with experimental groups. For example, during 2005 and 2006, we collaborated with Prof. Estrin's group in the analysis of mechanisms of ligand diffusion and reaction of *Mycobacterium tuberculosis* truncated-hemoglobin-N (Crespo *et al*, 2005; Bidon-Chanal *et al*, 2006). This enzyme is responsible for NO detoxification, and its overexpression leads to the resistance of *Mycobacterium tuberculosis* to macrophage action. In particular, during 2006 we characterised the main diffusion pathways for NO and O_2 in the enzyme, thereby locating the bottlenecks and control points of the process. An additional project, in collaboration with a pharmaceutical company, has led to the defi-

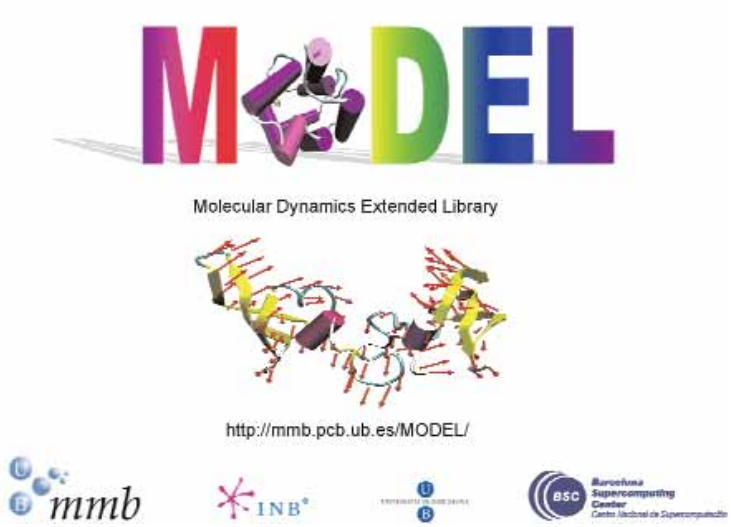

***Figure 1**. Main page of the MODEL webserver and database.The analysis of this massive database of trajectories gives a complete consensus picture of protein dynamics. The proteins are found to be melted solids (the core being solid-like and the surface being similar to a dense liquid). The essential dynamics of the proteins is clearly printed in their 3D structure, independently of the force-field and exact simulations conditions. Hydrogen bond interactions are the stiffer ones inside the proteins, while quite surprisingly, the saline bridge appears to be highly labile (Rueda* et al*, 2006). Overall, our study tested the capacity of force-field simulations to describe protein dynamics in physiological conditions.*

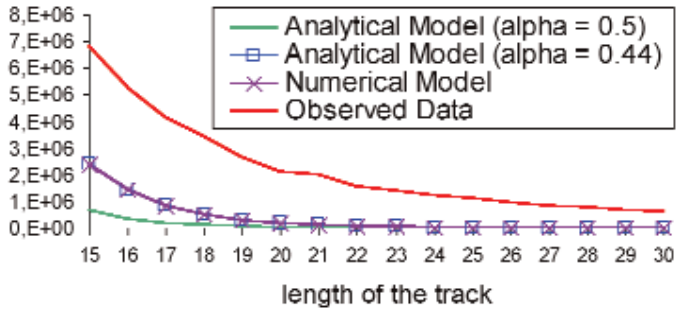

***Figure 2**. Representation of the number of nucleotides in triplex-forming oligonucleotides of different lengths compared with two background models.*

nition of the 3D recognition properties of a new family of $P_{38}\alpha$ MAP Kinase inhibitors which are now being tested as powerful anti-inflammatory compounds in phase II clinical trials (Soliva *et al*, 2006).

Since 2004 we have expanded our interest in proteins to the study of more general aspects of their structure and flexibility. The installation of the *MareNostrum* computer at the Barcelona Supercomputer Center (BSC-CNS) has opened up unexpected possibilities to perform proteome-scale studies. This is the origin of MODEL (Molecular Dynamics Extended Library; see Figure 1), the largest database of molecular dynamics simulations, currently containing information on the dynamics of around 1300 proteins (see http://mmb.pcb.ub.es/MODEL). The project has produced the first results, published during 2006 (Meyer *et al*, 2006 and Rueda *et al*, 2006a). The most remarkable of these studies is the definition of γMODEL, a restricted set of around 40 highly representative proteins which was simulated for 10 (some cases 100) ns using the four most commonly used force-fields (Rueda *et al*, 2006a).

Genome mining studies

Like many bioinformatics groups, our interest lies in obtaining information on key biological processes by means of the analysis of available biological databases. Our efforts have focussed on two directions: i) analysis of genomes to determine a pattern of DNAs with unusual structures and ii) development of predictive tools to determine the pathological character of single nucleotide polymorphisms.

Within the first project, during 2006, we have completed the mapping of TTS (triplex targeted sequences) in the human genome (Goñi *et al*, 2006). We have found that these triplexes are many times more common in the human genome than in background models (see Figure 2), and that they are over-concentrated in promoter regions, especially of those genes related to the control of cellular functions. These results open up exciting possibilities for triplex strategies, such as anti-gene therapies, since triplex formation at promoters are known to knock-out or knock-down genes.

We have continued to make progress in the analysis of the trends that determine when a mutation is neutral or pathological. Improvements in our PMUT server (http://mmb.pcb.ub.es/PMUT) have been made (see Figure 3). This server is now one of the most used for prediction of the pathological nature of SNPs. Furthermore, a comparative analysis on neutral, pathological and correlated mutations (Ferrer-Costa *et al*, 2006) has shown that each individual mutation of the correlated pair is characterised by a low pathogenicity index. This observation indicates that individual mutations can persist for long periods, without having a large impact on the species, before being stabilised by a complementary mutation (Ferrer-Costa *et al*, 2006).

Structural studies on unusual nucleic acids

This field has traditionally been one of great activity in the group (for a review see Orozco *et al*, 2003). During 2006 we have focussed our efforts on both the methodological aspects of analysis of the molecular dynamics trajectories of nucleic acids (NA) (Noy *et*

al, 2006) and on the detailed study of several unusual conformations. In particular, following previous studies (Cubero *et al*, 2003), we have explored the possibility of short fragments of Hoogsteen anti-parallel DNA embedded into long B-DNA duplexes. Extended molecular dynamics simulations demonstrate that, quite surprisingly, not only is the parallel Hoogsteen a stable structure, but the H-B junction does not introduce dramatic distortions into the overall structure of the helix. We also focus our attention on quadruplex DNA (G-DNA), a minor conformation of DNA that contributes to telomere stabilisation and is a major target for telomerase inhibition. We have reported for the first time that, in the presence of monovalent ions, G-DNA is so stable that it can survive to full vaporisation (Rueda *et al*, 2006b). If fully confirmed experimentally (available experimental information supports our hypothesis), G-DNA will be the first biopolymer to show stability in gas phase conditions, which would open up unexpected biotechnological applications for DNA quadruplexes, especially in the field of conductor design.

Another long-standing focus of our research efforts is anti-gene therapy. Here we seek to target a duplex DNA by means of a triplex-forming oligonucleotide (TFO), which binds to the major groove of the duplex generating a triplex. Once the triplex is formed the gene is knocked out, especially when triplex formation occurs in the promoter region. Considerable effort by several groups in our lab focuses on designing TFOs that lead to more stable triplexes. However, very often the formation of the triplex is hampered by that of an alternative tetraplex (see Figure 4). Part of our work during 2006 has addressed the design of nucleobase derivatives with the capacity to prevent tetraplex formation at the TFO. Our simulations indicate that excellent results can be obtained with 8-aminoguanine (Cubero *et al*, 2006). This result has been confirmed in agreement with experimental data.

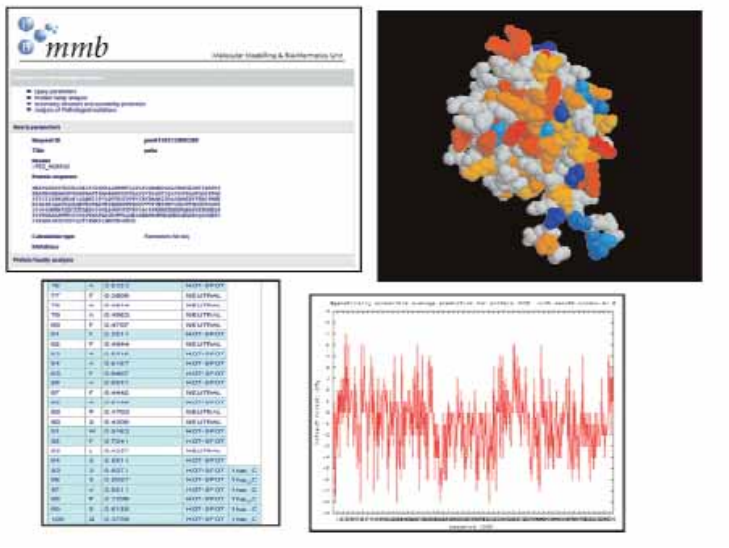

Figure 3. *PMUT(2006) Web server (http://mmb.pcb.ub.es/PMUT).*

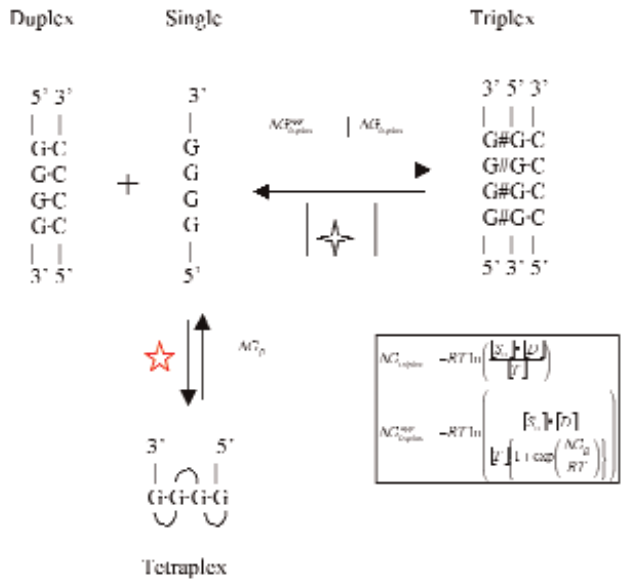

Figure 4. *Scheme of the inhibition of triplex formation by tetraplexes.*

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Modesto Orozco's group, March 2006.

Dynamic protein-protein and protein-ligand interactions in protein stability, gene regulation and drug design

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Dynamic intermolecular interactions are key elements in the regulation of many biological systems. While structural macromolecular complexes are usually stable and long lived, protein-protein interactions involved in regulation are dynamic. NMR is an especially powerful technique for the study of dynamic systems. The dynamic character can be the result of internal conformational dynamics in the constituent molecules, exchange processes involving alternative complexes or simply the equilibrium between free and bound forms. Examples of all the above categories are currently being studied by our group. Dynamic processes are potential candidates to be perturbed by small molecules as the energy difference between alternative states may be of the same order of magnitude as the binding energy of small ligands. Therefore, dynamic intermolecular interactions may yield drug targets of interest. Our group examines protein-protein interactions by NMR and searches for small molecules that can modulate these interactions in a therapeutically promising way. To this end, we develop our own methodology, including powerful computational and NMR screening tools. NMR is complemented by a variety of biophysical techniques to unravel complex protein-protein interaction problems, prior to attempting their modulation by small molecules. In addition, small molecules, or even Xe atoms, are being explored as reporters to characterise protein-protein interaction problems.

Interactions of nucleoid-associated proteins of the Hha and H-NS families

Up to 5% of the genome of *E. coli* is regulated by H-NS, an abundant nucleoid-associated protein. H-NS contains a DNA-binding domain and a dimerisation domain connected by what is known as the linker domain. Full length H-NS forms large molecular weight oligomers that interact with curved regions of DNA. Truncated forms of H-NS containing the first 46 or 64 residues form stable homodimers but do not undergo further oligomerisation. The Hha/YmoA family of nucleoid-associated proteins lack an intrinsic capacity to bind DNA but they act as co-regulators, along with H-NS, of the expression of several genes, including virulence factors such as hemolysin. In a very fruitful collaboration with Prof. Juarez's group, who discovered Hha, we have studied the interaction between Hha and truncated forms of H-NS. Our studies confirmed that Hha interacts with the N-terminal domain of H-NS in a complex process that involves a conformational rearrangement of the helices of Hha (García *et al*, 2005).

These observations prompted a study to examine a single cysteine residue in one of the loops connecting two of the Hha helices. Chemical modification and site mutations of this residue confirmed a key role of this interhelical loop. In fact, a designed mutant was shown to form *in vitro* complexes with H-NS that are more stable than those formed by the wild-type species. Introduction of this mutation in *E. coli* leads to a phenotype of disrupted capacity to grow at low salt concentrations. (Pons I, PhD dissertation UB, 2006; Cordeiro T, Research Report IST Lisboa; Cordeiro T *et al*, manuscript in preparation). Interestingly, it was discovered that Hha regulates gene expression in response to environmental changes such as variations in osmolarity and temperature.

Mutational studies in H-NS, followed by NMR studies of the interactions of the mutants with Hha, identified the primary Hha binding region and the identity of two key residues, whose mutation abolishes Hha binding, while preserving the structure of H-NS (Garcia *et al*, 2006). These mutants were used to

confirm that all relevant interactions with Hha occur only in the N-terminal domain of H-NS, as the introduction of a mutation in one of these residues in full length H-NS also abolished Hha binding.

The structural insight provided by the NMR studies was used to design a bioinformatic search for conservation in the relevant region of H-NS in a wide variety of Gram-negative bacteria. We found a clear correlation between sequence conservation and the presence of Hha paralogues, which was restricted to the *enterobacteriaceae* (Madrid *et al,* 2006).

The role of Hha/H-NS interactions in the regulation of osmolarity-induced changes was studied by following the formation of complexes at a range of salt concentrations. Two distinct complexes of different stoichiometry were formed at 100 mM and 150 mM NaCl and a sharp transition was observed between the two complexes, bracketing the physiological salt concentration (García *et al,* 2007).

Medium effects on protein stability and oligomerisation equilibria

Bovine low molecular weight phosphatase forms homodimers at high protein concentration. These homodimers, whose structure has been captured in crystals of a mutant, involve residues in the active site and are, therefore, functionally inactive. 15N-relaxation NMR studies had previously shown that at least a higher order oligomer (a tetramer) is formed. Our working hypothesis is that these oligomerisation processes are biologically relevant as regulatory mechanisms of the "recovery" stage of kinase mediated signalling events and that they are active at physiological concentrations under crowding conditions. We are currently studying conditions that are NMR friendly and that can mimic some aspects of crowding. We have characterised a mixture of charged aminoacids arginine and glutamate and shown that they induce homo-oligomerisation, while preventing non-specific protein association. As part of this effort, and in collaboration with E. Brunner's group in Regensburg, we are developing new methods to examine protein-protein association using Xe NMR.

We have also used the low molecular weight phosphatase as a test system for the new screening protocol developed by our group (see below).

Co-solutes, such as phosphate ions, may also have considerable effects on protein stability (Fayos *et al,* 2005). These effects are related to the modulation of the interaction of water with the protein surface and can be rationalised in terms of excluded volume effects, which increase protein stability, and of preferential interactions, which have a destabilising effect. This analysis provides an explanation for the Hofmeister effect. NMR chemical shift perturbations provide an independent experimental evidence of this analysis and differentiate between stabilising and destabilising ions (Tadeo *et al*, 2006). These studies were led in our group by Dr. Oscar Millet until his appointment as a PI in Bilbao and continue as an external collaboration.

Native unfolded proteins or the unfolded states of proteins are especially dynamic systems well suited for study by NMR and other biophysical techniques, such as Small Angle X-ray Scattering (SAXS). Dr. Bernadó maintains fruitful collaborations in this field using methodology he helped to develop during his stay in Grenoble. This research has resulted in several publications in PNAS and JACS, and was highlighted in *Science*.

Ligand screening of very large chemical libraries using structural information

An increasing number of drug-like molecules that are commercially available could be used in screening experiments. It has been recognised that a more efficient use of screening resources can be accomplished by virtual pre-screening. For targets of known structure, computational docking provides a ranking of ligands which can be critically assessed from the analysis of the predicted structure of the complex. Docking results provide the site specific interaction that matches that obtained experimentally by NMR. However, optimised docking algorithms cannot be used to screen typical databases containing millions of compounds.

This obstacle can be alleviated by combining docking to the target structure with similarity comparison between ligands. With this aim, we required a simi-

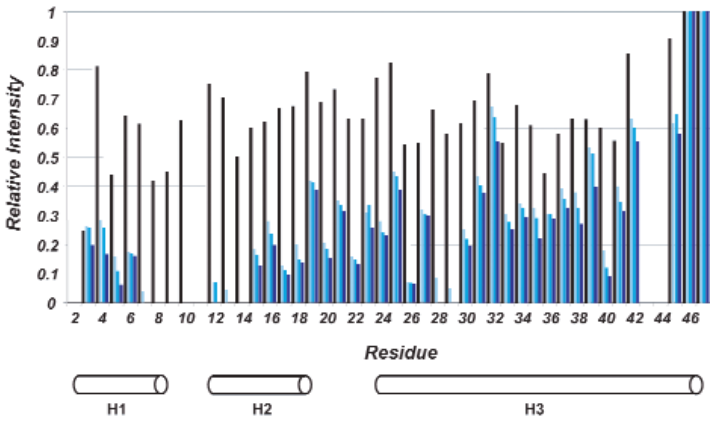

***Figure 1**. Mapping the Hha binding surface on H-NS by NMR.*

larity measure that captured the essential features that determine the activity of ligands and that could be computed with an extremely high efficiency. We turned to the most compact representation of a molecule that contains all the information needed to build a full 3D structure and to predict its intrinsic properties. This compact representation is simply its systematic chemical name: from this one dimensional string of characters a chemist, or a series of computer programmes, can reconstruct the 3D structure of the molecule. The LINGO concept, developed in our lab provides a way to analyse chemical names (IUPAC or SMILES) as textual objects, and to directly extract the implicit information they contain.

This method can be used to compute bioisosteric competent similarities at a speed of 75,000 comparisons per second and to predict properties at a speed of more than 120,000 molecules per second (Vidal *et al*, 2005).

We have integrated LINGO-based tools into a virtual screening protocol with the capacity to handle very large databases with minimal computational resources (Vidal *et al*, 2006). The method is conceptually a genetic algorithm but deals with complete individuals, not genes, and cross-over and heredity are implemented as similarity-based selection from a database. Genetic variability is implemented by random incorporation of a fraction of each new population. Despite the use of similarity measures, our results display good scaffold-hopping capacities (Figure 2).

We have checked this method experimentally using a low molecular weight phosphatase. From a database of half a million compounds, we selected 34 for experimental screening and nine of these had binding affinities that were better than those of previously described inhibitors (Vidal *et al*, submitted).

Experimental screening can be performed in several ways. NMR-based screenings produce fewer false positives than alternative methods and since they usually focus on binding, they are generally widely applicable, including systems with no associated enzymatic activity, such as protein-protein complexes. Screening based on chemical shift perturbations of the protein spectra requires the complete recording of 2D spectra. If the residues expected to be perturbed are known, it is possible to restrict the acquisition to the relevant frequencies with a substantial saving in experimental time using Hadamard spectroscopy (Feliz *et al*, 2006).

Some of the ligand screening methodology described is being successfully used in an industrial collaboration, subject to intellectual property restrictions and under the responsibility of Dr. Yolanda Pérez.

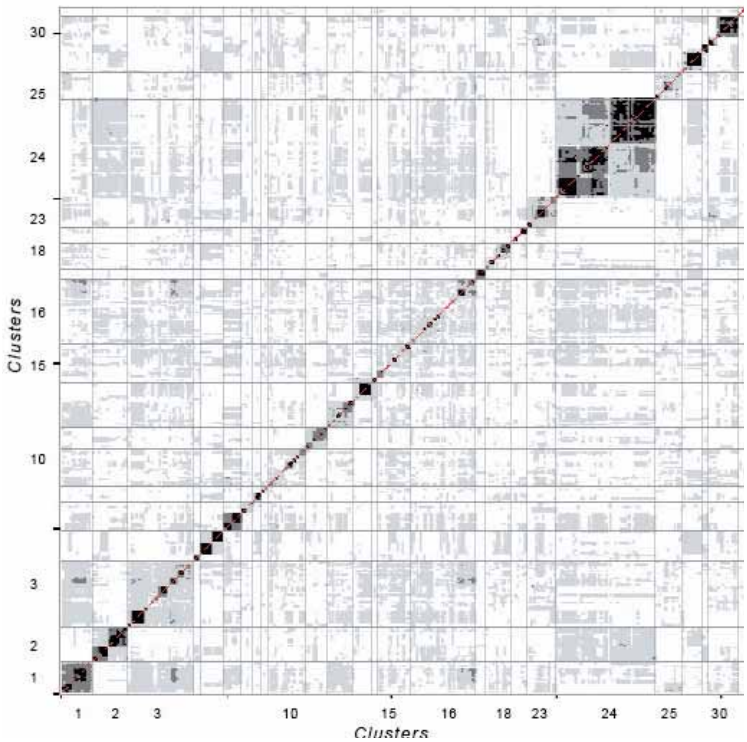

Figure 2. *Similarity matrix of the solutions obtained for inhibitors of Factor X protease.*

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RESEARCH NETWORKS AND GRANTS

Ligandos de STAT6 para el tratamiento del asma
Ministerio de Educación y Ciencia-FEDER-Laboratorios Uriach Proyecto PETRI: 2004-2006
Project coordinator: Miquel Pons

Nuevos métodos de RMN para el estudio de interacciones proteína-proteína
Ministerio de Educación y Ciencia-FEDER (BIO2004-5436): 2005-2007
Project coordinator: Miquel Pons

Genómica estructural: aplicación a proteínas y complejos proteicos relacionados con el cáncer
Ministerio de Eduación y Ciencia (subproject GEN2003-20642-C09-04): 2004-2007
Project coordinator: Miquel Pons
Subproject coordinator: Ernest Giralt

Non-covalent interactions between functional abiotic receptors and ion pairs
COST action D31:2005-2009
Network coordinator: A. Dalla Cort

Reference laboratory of the NMR facility of Barcelona. Ministerio de Educación y Ciencia
Scientific director: Miquel Pons

EMAR multidisciplinary frontiers of magnetic resonance
European Science Foundation. Approved by scientific committee, pending financial allocation: 2007-2012
Network coordinator: Miquel Pons

COLLABORATIONS

Structural studies of nucleoid associated proteins
Antonio Juarez (Faculty of Biology, University of Barcelona, Spain)

Use of Xe NMR to study protein-protein interactions and protein cavities
Eike Brunner (University of Regensburg, Germany)

Drug discovery based on computational docking and LINGO tools
Michael Thormann (Origenis AG, Germany)

Native unfolded proteins
Martin Blackledge (Institute de Biologie Structurale, Grenoble, France)

Solvent accessibility of unfolded proteins
Javier Sancho (University of Zaragoza, Spain)

Hydrodynamic studies and NMR
Jose García de la Torre (University of Murcia, Spain)

Surface effects on protein stability
Oscar Millet (CIC-BIOGUNE, Bilbao, Spain)

Discovery of modulators of STAT6 mediated signal transduction pathways
Lab Uriach, Spain

AWARDS

Tiago Cordeiro
Best Poster (Luso-Portuguese-Iberoamerican-NMR meeting 2005)

Pau Bernadó
Best Poster (III Bienal GERMN, Alicante 2006)

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Molecular Medicine Programme

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Cell signalling group

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The cell signalling group studies the regulatory and cross-talk mechanisms that underlie signal transduction. We focus on two main research lines: a) the cross-talk mechanisms and actions mediated by the interaction of members of the nuclear receptor superfamily and the JNK signalling cascade; and b) the pathway comprising the NIMA-family kinases Nercc1, Nek6 and Nek7.

Cross-talk mechanisms and actions mediated by the interaction of members of the nuclear receptor superfamily and the JNK signalling cascade

The c-Jun N-terminal kinase (JNK) is a group of serine/threonine protein kinases, which belongs to the mitogen activated-protein kinase (MAPK) family and is encoded by three genes (*jnk1*, *2*, and *3*), from which up to 10 isoforms are generated. Like other MAPKs, JNK participates in signal transduction as the last step of a MAPK module, in which two other protein kinases are involved. Along this module the signal is transduced as sequential phosphorylation and concomitant activation, of these protein kinases. JNK is activated by dual phosphorylation on threonine and tyrosine residues within the Thr-Pro-Tyr motif by a MAP2K (MKK7 or MKK4), which in turn has been activated by MAP3K-dependent phosphorylation (a large number of protein kinases participate in this step). JNK activation results in phosphorylation (and functional regulation) of its substrates, which are nuclear proteins such as the component of the AP-1 complex, c-Jun, or cytoplasmic proteins, such as the insulin receptor substrate (IRS)-1. JNK activity is negatively regulated by protein phosphatases such as the dual specificity MAP kinase phosphatase (MKP)-1 (revised in Kyriakis and Avruch, 2001). In general, JNK is activated by pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α or interleukin (IL)-1β, and stress agents such as ultraviolet light (UV), oxygen reactive species (ROS) or endoplasmic reticulum (ER) stress. The implication of JNK in a number of pathological conditions, such as cancer, cardiac hypertrophy and failure, arthritis, asthma, neurodegeneration, obesity and diabetes, has prompted an intensive search for JNK inhibitors for potential therapeutic use (revised in Manning and Davis, 2003).

In particular, our research centres the role of JNK in inflammatory response, obesity and type 2 diabetes, conditions that are linked. Insulin resistance, that is to say, the progressive loss of response to insulin by peripheral tissues, is an early condition in the development of type 2 diabetes. Afterwards, pancreatic β-cell failure definitively establishes the progression from insulin resistance to hyperglycemia, and thus the diabetic condition. In addition to genetic and environmental factors, obesity is the major risk factor for type 2 diabetes, and is strongly associated with insulin resistance. The connection between inflammation, obesity and insulin resistance was envisaged more that a decade ago after the observation that adipose tissue from obese mouse and humans overexpressed the pro-inflammatory cytokine TNF-α. Moreover, TNF-α treatment of cells or mice inhibits insulin action, and genetic ablation of the TNF-α gene or its receptor improves insulin sensitivity in the obese mouse. In fact, it is now accepted that a low-intensity inflammatory state is concomitant to obesity and is responsible for the overproduction of many pro-inflammatory mediators, which, like TNF-α, inhibit insulin action (revised in Wellen and Hotamisligil, 2005). In addition, in recent years it has been shown that the inflammatory response is activated by metabolic stress (since it induces stress in the ER, which eventually leads to the activation of the unfolded protein response (UPR)) or by excessive production of ROS, conditions that are promoted in obesity and contribute to insulin resistance. Pro-inflammatory cytokines, stress in ER or ROS, activate a set of serine/threonine protein kinases, such as JNK, IKK and PKC-∏, which in turn inhibit insulin signalling (revised in Hotamisligil, 2005).

Compelling evidence supports a role of chronic inflammation in the development of pathologies such as cancer, obesity and diabetes, a relationship that explains the beneficial effects of drugs with anti-inflammatory activity in these diseases. In this regard, several ligands of nuclear receptors (NRs), such as glucocorticoids (GCs) and the ligands for the peroxisome proliferator-activated receptors (PPARs) and liver X receptor (LXR) show anti-inflammatory properties. At the molecular level, this pharmacological action is due to the capacity of their receptors (GR, PPARs and LXR) to antagonise the transcriptional regulators AP-1 and/or NF-κB, which are responsible for orchestrating the inflammatory response (revised in Glass and Owaga, 2006). Several mechanisms have been proposed to mediate this antagonism and, among others, our research group has described the inhibition of the JNK pathway by GCs as a mechanism responsible for the interference of these hormones with the AP-1 complex (Caelles *et al*, 1997; Caelles *et al*, 2002). In recent years, we have gained insight into the molecular mechanisms that mediate GC interference with the JNK pathway. In this regard, this interference can be conducted by transcription-independent and -dependent mechanisms. The former involves the direct and hormone-induced GR-JNK interaction through a MAPK docking site located at the N-terminus of the ligand binding domain of the GR, and explains why transactivation-deficient mutants of GR are competent to inhibit the JNK pathway and, consequently, to antagonise the AP-1 complex (Bruna *et al*, 2003; Figure 1). Regarding the transcriptional-dependent mechanism, it has been shown that the mkp-1 gene is a transcriptional target of GCs (Kassel *et al*, 2001).

PPARs and LXR have crucial roles in lipid and glucose metabolism as well as in macrophage homeostasis. PPARs are a subfamily composed by three members, PPARα, PPARδ and PPARγ, which, although with some overlap, show specific expression patterns and specificity to their ligands. In relation to the latter, natural ligands for PPARs have been identified and are molecules derived from lipid metabolism; synthetic ligands have also been obtained and show relevant pharmacological properties such as TZDs, which are PPARγ ligands with insulin sensitising action.

In this context, our interest and, thus, the main goal of our research is to study the physiological actions of GCs and TZDs mediated by interference with the JNK pathway (essentially in the context of inflammation, obesity and type 2 diabetes) as well as, the molecular mechanisms responsible for this interference *in vivo*.

GCs may also inhibit JNK by the transcriptional activation of the mkp-1 gene. In this regard, using either bone marrow or thioglicollated-elicited peritoneal macrophages derived from mkp-1 knock out (KO) mice, we have shown that GCs rely on MKP-1 for their capacity to inhibit pro-inflammatory gene expression as well as to inhibit the LPS-induced activation of JNK and p38 MAPKs. Nonetheless, GC anti-inflammatory activity *in vivo*, analysed either in local inflammato-

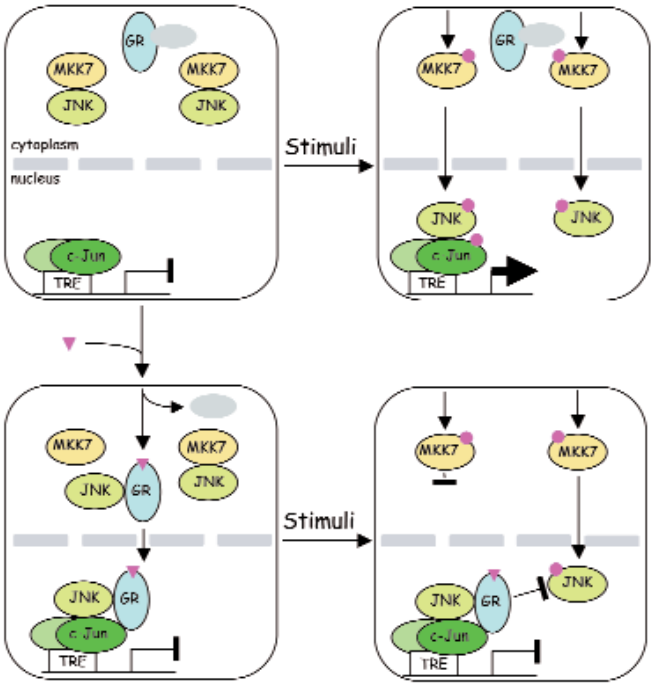

***Figure 1**. GC action on the JNK pathway. (1) In the absence of GCs, GR is located in the cytoplasmic compartment and associated with multiprotein complexes. Likewise, in non-stimulated cells, JNK associated with MKK7 is tied by scaffold proteins into specific signalling modules. In the nuclear compartment, AP-1 complexes containing c-Jun are bound to the TPA response elements (TREs) found in the regulatory sequences of the AP-1 target genes, although the AP-1-dependent transcription is turned off. (2) Upon stimulation, the signal is transduced along the JNK pathway signalling modules by a cascade of phosphorylation events (filled circles) that ends in the activation of JNK. Active JNK dissociates from the signalling module and translocates into the nuclear compartment where it associates with and phosphorylates the c-Jun N-terminal domain. Thereafter, c-Jun phosphorylation induces the dissociation of a repressor complex resulting in AP-1 activation and, in consequence, triggers transcription of AP-1-responsive genes. (3) In the presence of GCs (inverted triangles), hormone-bound GR dissociates from the multiprotein complexes and exposes a JNK docking site. Some JNK molecules dissociate from the signalling modules, bind to GR and travel together with GR into the nuclear compartment where they associate with c-Jun. (4) In these conditions, upon stimulation, JNK-deficient signalling modules fail to transduce the signal, resulting in the GC-induced inhibition of JNK pathway activation. In consequence, fewer molecules of active JNK are produced and, hence, enter into the nucleus. In addition, these active JNK molecules have to compete for the c-Jun docking sites with the already c-Jun-bound inactive JNK molecules.*

ry or in LPS challenge tests, does not appear to require MKP-1, since GC action in the MKP-1 KO mouse does not impaired but only requires higher GC doses, indicating a certain degree of GC resistance in this animal model (Ferré S and Caelles C, manuscript in preparation).

In relation to the PPARs, we have shown that PPARγ ligands, including the insulin sensitising drugs TZDs, also have the capacity to down-regulate the JNK pathway. We have reported that the TZDs rosiglitazone and troglitazone inhibit TNF-α-induced JNK activation in 3T3-L1 adipocytes. Our results indicate that PPARγ mediates this inhibitory action since a) it is reproduced by other chemically unrelated PPARγ ligands and blocked by PPARγ antagonists; b) it is enhanced by PPARγ overexpression; and c) it is abrogated by PPARγ RNA interference (RNAi). In addition, we have demonstrated that rosiglitazone inhibits JNK activation and promotes the survival of pancreatic β-cells exposed to IL-1β. Moreover, *in vivo*, rosiglitazone inhibits the abnormally elevated JNK activity in peripheral tissues in two distinct murine models of obesity. Finally, we have shown that the hypoglycemic action of this drug is abrogated in the diet-induced obese *JNK1*-deficient mouse. In summary, we have described a novel mechanism, based on targeting the JNK signalling pathway, which is responsible for insulin-sensitising and, potentially, for the protective actions of TZDs/PPARγ on pancreatic β-cells (Díaz-Delfín *et al*, 2007).

To evaluate the interference of JNK by nuclear receptors *in vivo*, we have recently generated a transgenic mouse model harboring a Cre recombinase conditioned expression gene encoding a constitutively activated mutant of the MAP2K of JNK, MKK7 (GFP/MKK7D mice). The recombination/activation of this transgene induced by crossing these mice with those expressing the Cre recombinase in a tissue specific manner will specifically activate JNK in those targeted tissues. This transgenic model shows a good level of expression, as shown by detection of the green fluorescent protein (GFP) in most of the tissues analysed. To test the proper activation of the transgene, as well as whether its activation leads to a predictable phenotype, we have performed several preliminary analyses in this transgenic mouse model. Homozygous GFP/MKK7D mice were crossed with mice harboring a Cre recombinase transgene specifically expressed in pancreatic β-cells (strain B6.Cg-Tg (ins2-cre25)MgnJ from The Jackson Laboratory). In accordance with our prediction, based on data from the literature showing a role of JNK in pancreatic β-cell death, those mice in which the trangene had been activated showed defects in glucose metabolism in adulthood, as demonstrated by the glucose tolerance test. Together with *mkp1*- and *jnk1*-deficient mice that we already have in our laboratory, this transgenic mouse will be a basic tool to study the actions of the above-mentioned nuclear receptors ligands mediated by interference with the JNK pathway in the whole animal.

Finally, in collaboration with other groups, we have participated in studies related to the biological actions mediated by MAPK pathways, such as the ERK pathway in *Drosophila* (Astigarraga *et al*, in press), the p38 MAPK pathway in myogenesis (Perdiguero *et al*, *2007*) and JNK in macrophages (Sanchez-Tilló *et al*, 2007).

The pathway comprising the NIMA-family kinases Nercc1, Nek6 and Nek7

Protein kinases are central elements in the regulation of the processes that lead to the division and multiplication of eukaryotic cells. Several protein kinase families are responsible for the spatio-temporal organisation of the myriad of events that allow a cell to divide and thus produce two daughter cells. $p34^{Cdc2}$/CDK1, the protein kinase that initiates mitosis, collaborates in the execution of this phase of the cell cycle with other mitotic kinases, mostly belonging to the Polo and Aurora families, and to the lesser studied NIMA family (designated *Nek* in vertebrates; Nigg, 2001). The NIMA/Nek family is composed by the protein kinases that are evolutionarily related to the *Aspergillus* NIMA kinase, an enzyme with the unique characteristic of being required for mitotic entry, independently of the activation state of $p34^{Cdc2}$, and that play a role in the organisation of the nuclear envelope and the spindle during mitosis (O'Connell *et al*, 2003). Eleven Neks have been described in mammalian cells, and it has been proposed that the expansion of the Nek family reflects a subdivision of the functions of NIMA among a divergent array of related kinases, and perhaps also the introduction of new responsibilities. A number of experimental results indicate that at least some of these functions are related to the control of the structures formed by centrosomes and microtubules, such as the mitotic spindle during mitosis and, in superior eukaryotes, cilia and flagella (Quarmby and Mahjoub, 2005). A detailed study of the regulation and function of Neks is required to elucidate the precise role of each family member.

We are studying three members of the Nek family and their involvement in the regulation and execution of mitosis: Nercc1/Nek9 and its downstream kinases, the highly homologous Nek6 and Nek7 (Roig *et al*, 2002; Figure 2). Nercc1 activation during mitosis occurs in parallel with Nek6 (and perhaps Nek7) binding, resulting in Nek6/7 phosphorylation and subse-

quent activation (Belham *et al*, 2003). Interference with Nercc1 impedes mitotic progression, and specifically results in spindle abnormalities, followed by prometaphase arrest or aberrant chromosome segregation and thus aneuploidy and centrosome amplification (Roig *et al*, 2002). Recently, Nercc1 activation has been localised to the centrosomes and poles of the mitotic spindle, and shown to be required for the correct formation of the mitotic spindle (Roig *et al*, 2005). Cells in most late-stage cancers are aneuploid, genetically unstable, and show distinct degrees of centrosome amplification; centrosome and spindle abnormalities and aneuploidy have also been detected in early-stage carcinomas, and thus have been proposed as a major factor in the multi-step establishment of the transformed phenotype of cancer cells (through, *e.g.*, loss of heterozygosis of tumour suppressor genes). Therefore, we hypothesise that Nercc1, Nek6 and Nek7 dysfunction is involved in the molecular processes that lead to transformation and cancer. A greater understanding of the regulation and function of the Nercc1/Nek6/7 signalling pathway is required to test this hypothesis, as well as to determine whether the specific inhibition of the protein kinases could be used therapeutically as an antiproliferative strategy.

The pathway comprising the NIMA-family kinases Nercc1, Nek6 and Nek7

To understand the physiology of the Nercc1/Nek6/7 signalling *cassette*, we are currently starting studies on its upstream regulation as well as a search for downstream substrates that would explain the cassette's central role in the control of the structure and function of the mitotic spindle. Our long term goal is to elucidate the position of the kinases among the mitotic signalling pathways and downstream effectors.

We have performed a two-hybrid screen to identify proteins that bind to the domains of Nercc1; we are analysing the interactors identified to determine their relevance in the regulation of Nercc1 activity, or as substrates of the kinase or its associated partners Nek6/7. We have previously characterised the *in vitro* mechanism of Nercc1 activation, and determined that isolated Nercc1 autoactivates by autophosphorylation at subcellular concentrations of ATP. These results indicate a mechanism *in vivo* that inhibits or reverts this process. We are currently examining the role of the Nercc1 interactors in this mechanism. The role of the observed phosphorylation and subcellular localisation of Nercc1 during mitosis is also under study.

In addition to our search for novel interacting proteins, we are also studying two known Nercc1/Nek6/7 interactors and substrates by characterising their phosphorylation by kinases and the relevance of this modification on the control of their physiological activity.

Given that the inhibition of mitotic signalling can be used therapeutically as an anti-oncogenic treatment, in collaboration with the biotechnology company Mercury Therapeutics, Inc (Woburn, MA, USA), we are currently performing an identification programme for Nerc1 inhibitors using methods protected by a patent coauthored by J Roig. A number of candidates, resulting from a screening of several libraries comprising ~100,000 compounds, some with strong anti-mitotic effects, are now being evaluated and characterised in the lab. In parallel, in order to better understand the function of these kinases and to validate its chemical inhibitors, by means of siRNAS, we are currently examining the effects of interfering with Nercc1, Nek6 and Nek7.

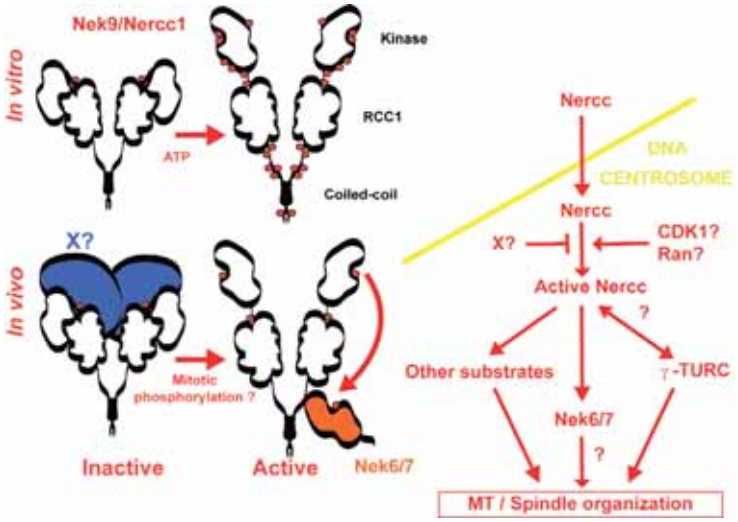

***Figure 2**. Left: a model for the* in vitro *and* in vivo *mechanisms of Nercc1 activation. Red circles indicate known phosphorylation sites. Right: the Nercc1/Nek6/Nek7 signalling cassette. Some of the possible upstream regulators and downstream substrates are depicted.*

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RESEARCH NETWORKS AND GRANTS

Mecanismos de interferencia de los glucocorticoides con las rutas de MAPKs como base de su acción anti-inflamatoria. Potenciación de la acción antiinflamatoria de los glucocorticoides actuales
(reference 030910)
Fundació La Marató de TV3, Spain: 2004-2006
Project Coordinator: Carme Caelles

Estudio in vitro *e* in vivo *de la interacción de los glucocorticoides y la ruta de la Jun N-terminal kinase (JNK): Mecanismos y acciones fisiopatológicas y farmacológicas mediadas por esta interacción*
(BFU2004-02096). Plan Nacional I+D+I 2004-2007,
Ministerio de Educación y Ciencia, Spain: 2005-2007
Project Coordinator: Carme Caelles

Estudio de una nueva vía de señalización mitótica compuesta por las NIMA quinasas Nercc1, Nek6 y Nek7. Regulación y funciones
(RYC-2004-001074). Programa Ramón y Cajal
Ministerio de Educación y Ciencia, Spain: 2005-2007
Project Coordinator: Joan Roig Amorós

Estudio de una nueva vía de señalización mitótica compuesta por las NIMA quinasas Nercc1, Nek6 y Nek7. Regulación y funciones
(BFU2005-05812)Plan Nacional de I+D+I,
Ministerio de Educación y Ciencia, Spain: 2006-2008
Project Coordinator: Joan Roig Amorós

A novel mitotic signalling pathway composed by the NIMA-family protein kinases Nercc1, Nek6 and Nek7. Regulation, functions and possible implication in cellular transformation
(MIRG-CT-2005-031088) EU Marie Curie International

Reintegration Grants: 2006-2007
Project Coordinator: Joan Roig Amorós

Consolidated Research Group
2001SGR00432 Generalitat de Catalunya: 2004-2007
Project Coordinator: Diego Haro

COLLABORATIONS

MAPK signalling pathways in myogenesis
Pura Muñoz (Centre de Regulació Genòmica, CRG, Barcelona, Spain)

Downstream effectors of the Nercc1/Nek6/7 signalling cassette
Joan Roig Amorós (IRB Barcelona, Spain)
Joseph Avruch (Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA)

MAPK signalling pathways in macrophage
Antonio Celada (IRB Barcelona, Spain)

Nercc1 inhibitors and their potential as antimitotic drugs
Joan Roig Amorós (IRB Barcelona, Spain)
Mercury Therapeutics, Inc (Woburn, MA, USA)

AWARDS

Carme Caelles
Distinció de la Generalitat de Catalunya, 2002-2006

Carme Caelles' group, March 2006.

Biology of macrophages: Regulation of gene expression

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Consol Farrera
Maria G Sans

Antonio Celada

Our project is the continued work of many years devoted to the biology of macrophages and dendritic cells. These cells play a key role in the innate immune response, and form a bridge between innate and acquired immune response. They are involved in the pathogeny of a large number of chronic inflammatory diseases (such as rheumatoid arthritis) and autoimmune diseases. Most immune cells act during the initial stages of microbial aggression on the organism. In contrast, macrophages are also involved in the late phases of the inflammatory process and they survive in tissues for a long time, until inflammation disappears.

Macrophages are generated in bone marrow and reach all body tissues through the blood. In normal conditions, few cells are differentiated in response to certain chemokines and become mature cells or tissue specific cells: dendritic cells, Kupffer cells, microglia, *etc*, while most are eliminated by apoptosis. When an inflammatory process is produced, macrophages proliferate, differentiate or become activated under the effect of other chemokines or growth factors. When a macrophage becomes activated, it ceases to respond to proliferative stimuli. In certain circumstances, when chronic inflammation is produced, macrophages have a harmful effect, causing lesions. For this reason, macrophages exert positive effects during acute inflammation, but deleterious effects in some chronic inflammations.

One of the most intriguing points in our field of study comes from the fact that we use non-transformed cells which respond to stimuli that induce proliferation (M-CSF, GM-CSF, IL-3), differentiation (GM-CSF), activation (IFN-γ, LPS, IL-4, IL-10, IL-13) or apoptosis. Our findings contradict several observations previously made in cell lines, most of which do not depend on any growth factors to proliferate.

Signal transduction and gene regulation mediating proliferation, activation and apoptosis of macrophages

One of the best characterised signal transduction pathways is involved in the sequential activation of Ras, Raf-1, mitogen/extracellular signal-regulated kinase (MEK) and the extracellularly regulated kinase (ERK). Activated Raf-1 phosphorylates MEK1 and MEK2 kinases, which in turn activate ERK1 and ERK2. In unstimulated cells, ERK1 and ERK2 are found in the cytoplasm and they relocate to the nucleus after being phosphorylated. Once in the nucleus, they phosphorylate a series of transcription factors. These kinases also participate in the synthesis of nucleotides and in protein translation processes, both required for proliferation and cellular activation. We have observed that ERK activation is required not only for proliferation, but also for LPS-mediated activation, although this activation also blocks proliferation. The duration and time of initiation of ERK phosphorylation determines whether the cell proliferates (short phosphorylation) or becomes activated (long phosphorylation). This is explained by the fact that MKP-1, the phosphatase responsible for ERK dephosphorylation, is induced rapidly in response to M-CSF or slowly in response to LPS. In both cases, MKP-1 induction is mediated by PKCμ and is independent of ERK phosphorylation. We have also reported that Raf-1 activation is necessary for this induction and is also mediated by PKCε (Sánchez-Tilló *et al*, 2006a). Furthermore, IFN-γ, which also inhibits proliferation, blocks MKP-1 induction by M-CSF by elongating ERK phosphorylation (Valledor *et al*, submitted). Inhibition of MKP-1 induction by RNA interference (RNAi) blocks proliferation and elongates ERK activation.

We have cloned the MKP-1 promoter, and by means of luciferase mutations and activity assays, we have localised an AP-1/CRE box which is critical for MKP-1

induction by M-CSF and by LPS. By Electrophoretic Mobility Shift Assays and Chromatin Immunoprecipitation, we have determined that this box is bound by Jun and CREB factors. c-Jun is induced by LPS and M-CSF with the same kinetics as MKP-1 (Casals *et al*, submitted). Finally, these factors induce JNK (c-Jun-N-terminal kinase) activation, which is required for MKP-1 induction (Sanchez-Tilló *et al*, 2006a,b; Figure 1).

Macrophage proliferation is independent of calcineurin (Comalada *et al*, 2003a), but requires immunophilin, without which ERK is inactivated (Sanchez-Tilló *et al*, 2006a,b). Both M-CSF-induced proliferation and IFN-γ– or LPS-induced activation requires RNA synthesis. Nevertheless, macrophages use several nucleoside transport systems for proliferation or activation. Nucleoside transporters N1 and N2 are induced by IFN-γ independently of Stat1. M-CSF induces the opening of K+ channels, which are required for proliferation (Vicente *et al*, 2005; Villalonga *et al*, 2007).

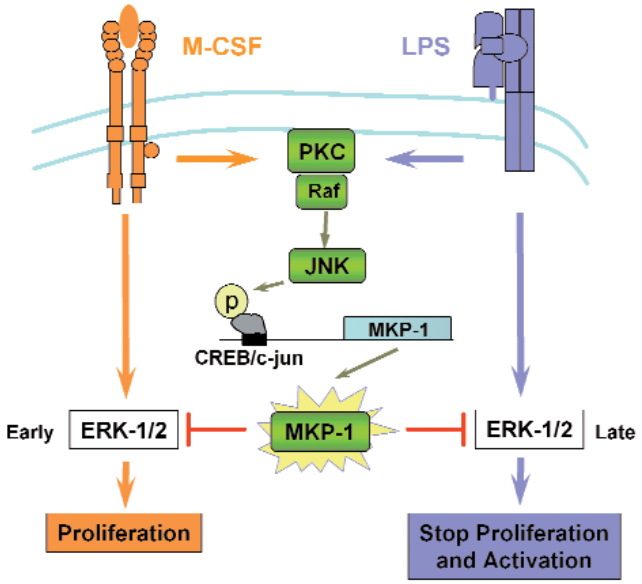

***Figure 1**. Signal transduction of M-CSF and LPS inducing the expression of MPK-1.*

Our group has devoted many years of research to the study of the regulation of MHC class II molecules. Peptides derived from processed proteins bind to a cleft in the MHC class II molecule surface and are presented to T lymphocytes. Thus, the expression of MHC class II molecules regulates not only the generation of the T lymphocyte repertoire, but also the induction and maintenance of immune response.

MHC class gene transcription depends on the interaction and co-operation of several transcription factors which bind to the regulatory elements found in the promoter. However, all the transcription factors described to date show ubiquitous expression, which does not correlate with the differential tissue expression of MHC class II. A transactivator which does not bind directly to DNA, CIITA (class II transactivator), has been described and shown to be required for the expression of these genes. We have determined that an AP-1 box acting as an enhancer is responsible for the induction of expression in B lymphocytes and dendritic cells treated with LPS (Casals *et al*, submitted).

Molecular mechanisms that determine classical versus alternative activation of macrophages

Classical activation of macrophages is induced by IFN-γ or LPS (M1) while the activation triggered by IL-4, IL-10 or IL-13 is known as alternative activation (M2). Apart from a series of structural and functional modifications, the main difference between these phenotypes is the biochemical pathway used for processing aminoacid arginine. IFN-γ or LPS induce NOS2 enzyme, which produces nitric oxide (NO). NO has great destructive power, and in the first phases of inflammation kills microorganisms. In M2 macrophages, arginase is induced and produces proline and polyamines, which catalyse the reconstitution of the damaged extracellular matrix, an event that occurs during the final phases of inflammation. The entry of arginine to the cell is produced through specific transporters. We have seen that both classical and alternative activation of macrophages induce the expression of the CAT2 transport system in order to enhance arginine import (Yeramian *et al*, 2006a; Martin *et al*, 2006). This transport system limits macrophage activation. Arginine is also required for cell proliferation. In this case, it is introduced in the cell by the CAT1 transport system and, once inside, the cell it is not degraded but used mainly for the synthesis of new proteins (Yeramian *et al*, 2006b; Figure 2).

During collaboration with the groups led by Ingrid Muller (Imperial College London) and Manuel Modolell (Max Planck Institute, Freiburg), we have observed that susceptibility to Leishmania correlates with the production of arginase and polyamines, which parasites require for growth (Kropf *et al*, 2005).

Role of TREX1 exonuclease in transcription

We have cloned a protein that binds to DNA and corresponds to TREX1 exonuclease. This enzyme catalyses the digestion of DNA in the 3´->5´ direction and shows homology to the TREX2 exonuclease (30%). Genetically modified mice, with a deletion in the TREX1 locus, developed inflammatory myocarditis

and had a reduced half life compared to their wild type counterparts. In humans, mutations in the Trex1 gene have been associated with Aicardi-Goutières Syndrome, a chronic inflammation of the brain (Crow *et al*, 2006). TREX1 has also been associated with protein members of the SET complex, which digest DNA from cells where apoptosis has been induced by Granzyme A.

In collaboration with IRB Barcelona experts in crystallography (Ignasi Fita) and in NMR (Maria Macías), we have determined the structure of TREX1 alone and its binding to DNA (Brucet *et al*, 2007; Figure 3). TREX1 to binds preferentially to certain DNA sequences that correlate with the exonuclease activity. TREX1 has a proline-rich domain not found in TREX2. This domain allows interaction with SH3 or WW domains, which we have demonstrated by NMR and co-immunoprecipitation. These data and the nuclear localisation of the protein, have led us to study whether TREX1 is involved in transcription.

Deregulated gene expression in aging

We have been testing the molecular changes that occur in the genome of macrophages during aging. As we grow macrophages alone *in vitro*, we disregard the effects that other cells could exert on them

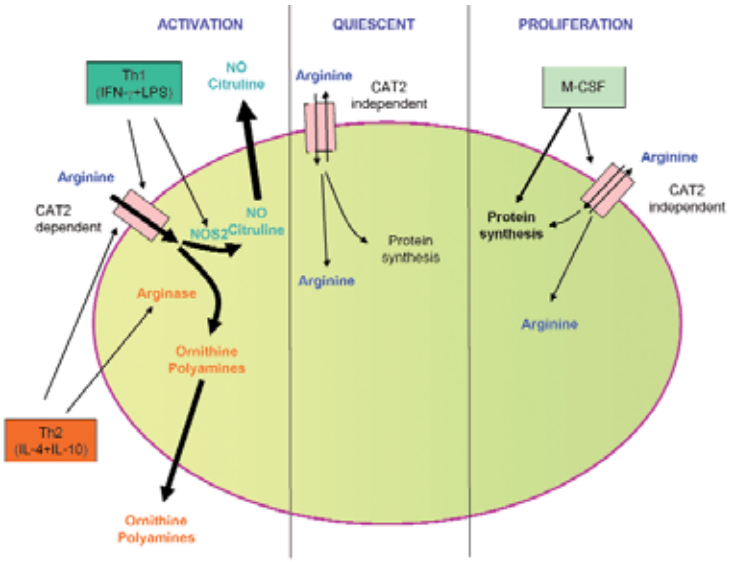

Figure 2. *Mechanisms of arginine uptake during activation, quiescence and proliferation.*

In addition, we have recently reported that deacetylase activity is required for GM-CSF-dependent functional response of macrophages and dendritic cell differentiation (Sebastian *et al*, submitted). Since deacetylase activity plays an important role in aging in lower organisms, these results have prompted us to study whether it is involved in macrophage aging.

LXR in neuroinflammation and neuronal degeneration (Annabel F Valledor)

LXRs (liver X receptors, initially discovered in the liver) are members of the nuclear receptor superfamily. Nuclear receptors are ligand-dependent transcription factors that regulate many aspects of development and homeostasis. LXRs are regulated by oxidised forms of cholesterol (oxysterols) and by intermediary products of cholesterol biosynthesis. At the physiological level, LXRs play a crucial role in the positive regulation of genes involved in lipid homeostasis.

We seek to explore whether activation of LXRs exerts anti-inflammatory and neuroprotective actions in the central nervous system (CNS). Neurons, as a result of their lack of cellular division and their low capacity to recover from injury, are extremely sensitive to inflammatory processes and immune autodestruction. For this reason, intervention of the inflammatory process has recently gained attention as a therapeutic strategy to halt neurodegenerative disorders (Valledor, 2005). Our preliminary studies show that both primary microglia and the microglial cell line BV-2 express the LXRα and β, and RXRα and β isoforms. In BV-2 microglial cells, stimulation with endogenous ligands of LXR, resulted in activation of known LXR target genes involved in lipid metabolism. In the mature brain and under physiological conditions, resting microglia serves the role of immune surveillance and host defence. However, these cells are particularly sensitive to changes in their microenvironment and readily become activated in response to infection or injury. Most of the factors released by activated microglia are pro-inflammatory and neurotoxic, thereby contributing to the progression of the neurodegenerative disorder. We have therefore studied the role of LXR in the regulation of microglial activation *in vitro*. Our results indicate that a number of pro-inflammatory factors are inhibited by LXR agonists. On the basis of these preliminary studies, we are currently using microarray technology to establish a more extensive list of pro-inflammatory genes susceptible to down-modulation by LXR agonists.

Furthermore, we have also explored the role of the LXR-RXR pathway on programmed cell death in the CNS. The simultaneous use of LXR and RXR agonists

resulted in synergistic effects that promote high expression of genes involved in protection against apoptosis in microglial cells, *eg*, Blc-xL, AIM and NAIP (neuronal apoptosis inhibitory protein). The observation that AIM, an anti-apoptotic factor secreted by macrophages, is also induced in this system leads us to propose that this factor mediates paracrine anti-apoptotic actions on other neighbour cells in the CNS, *eg*, astrocytes and neurons. We are currently testing these effects using mixed glia-neuronal cultures and pure neuronal systems. Microarray experiments in pure neuronal cultures are also underway in order to determine direct effects of LXR agonists on neuronal cells. Our final goal is to establish whether the anti-apoptotic and anti-inflammatory actions of the LXR-RXR pathway can be exploited for the therapeutic intervention of neurodegenerative disorders *in vivo*.

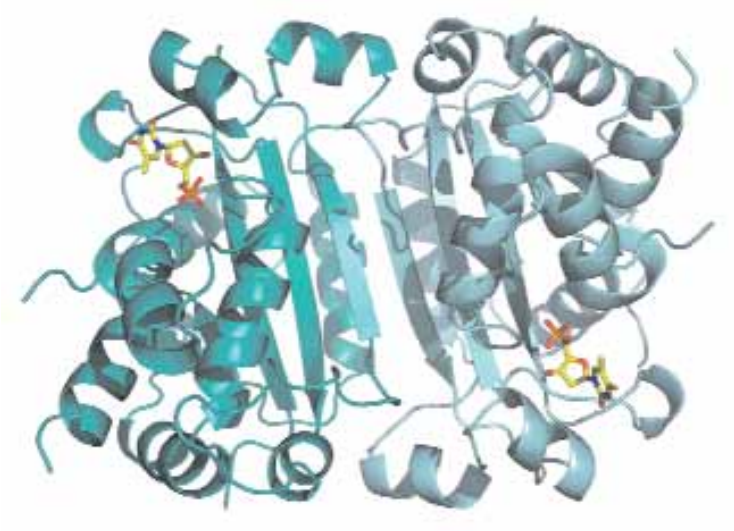

***Figure 3**. Three-dimensional structure of TREX1.*

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RESEARCH NETWORKS AND GRANTS

Regulation of the expression of genes involved in proliferation, differentiation, activation and apoptosis of macrophages and dendritic cells
Plan Nacional, Ministerio de Educación y Ciencia (BFU2004-05725/BMC): 2005-2007
Project Coordinator: Antonio Celada

Nanoparticles as activators of phagocytic cells for the cleareance of toxic aggregates of proteins in the brain
Ministerio de Educación y Ciencia (SAF2006-26676-E): 2006-2007
Project Coordinator: Jorge Lloberas

Anti-inflammatory and anti-apoptotic effects of LXR/RXR agonists in the central nervous system
European Commission-Marie Curie International Reintegration Grants (031137): 2006-2008
Project Coordinator: Annabel Fernandez Valledor

COLLABORATIONS

Macrophage activation and channels
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Neutrophils and apoptosis
Victor Asensi (Hospital General, Asturias, Spain)

Arginine and macrophages
Manuel Modolell (Max Planck Institute, Freiburg, Germany)

Arginine and macrophages
Carol MacLeod (University of San Diego, USA)

Leishmania and arginase
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Arginase and neutrophils
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Aging and polymerases
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Cell cycle and E2F1/2
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Structure of TREX1
Ignasi Fita (IRB Barcelona, Spain)

Structure of TREX1 and Itch
Maria Macías (IRB Barcelona, Spain)

Signal transduction and MAP-1
Carme Caelles (IRB Barcelona, Spain)

Macrophages and nanotechnology
Ernest Giralt (IRB Barclona, Spain)

Antonio Celada's group, March 2006.

Metabolic engineering and diabetes therapy

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Our group is involved in several research projects on glycogen metabolism, and its alterations in diabetes and Lafora disease (LD). In addition, a second line of research, related to the first, addresses the discovery and characterisation of compounds with anti-diabetic properties. Studies on glycogen metabolism have allowed the identification of many enzymes and intermediate metabolites involved in the synthesis and degradation of this polysaccharide. However, new factors and processes that participate in glycogen regulation are constantly being discovered. Moreover, data on the mechanisms of control in distinct organs and in diverse physiological conditions are incomplete. The alteration of one of these mechanisms may lead to serious pathologies such as diabetes mellitus and LD. The discovery of compounds that counteract the pathological alterations of glucose metabolism are of potential interest for the treatment of diabetes mellitus. Our group has demonstrated changes in the subcellular organisation of the enzymes that participate in glycogen metabolism, depending on the metabolic state of the cells. These changes in localisation represent an additional control mechanism of enzymatic activity, and are probably regulated by post-translational modifications. In addition, we have reported clear differences between muscle an hepatic glycogen metabolism. These differences are based on the distinct subcellular localisation of the glycogen synthase (GS) isoenzymes. We have recently resolved the 3D structure of the smallest known member of the GS family (*Pyrococcus abyssi* GS). This result has allowed the modelling of mammalian GS structures, a first step prior to the resolution of their 3D structure. Finally, we are currently characterising the molecular targets of an anti-diabetic and anti-obesity compound discovered by our group. Phase I of clinical trials with this compound has recently finished, and Phase II is about to start.

Regulation of hepatic, muscular and neuronal glycogen metabolism. Alterations in pathological conditions: diabetes mellitus and Lafora disease

Identification and functional analysis of the phosphorylation sites in the liver isoform of glycogen synthase

Nine phosphorylation sites, relevant for the activation of the muscle isoenzyme of glycogen synthase (MGS) have been described. A comparative sequence analysis showed that the liver isoenzyme (LGS) has 7 of these potential phosphorylation sites, two close to the N-terminus (named 2 and 2a) and five close to the C-terminus (named 3a,3b,3c, 4 and 5).

To progress on the study of the functional consequences of phosphorylation in these sites, we have generated recombinant adenoviruses with serine to alanine mutations on each of these residues (mimicking the non-phosphorylated state); and combined mutations of the N and/or C-terminal phosphorylation regions. Our results show that LGS mutated in a particular site increases its activation state, in contrast to the results published for MGS, for which none of the single mutations increased the activity. In addition, the combined mutation of this site with other phosphorylatable residues exerts a synergistic effect thereby greatly increasing LGS activation state and inducing the accumulation of glycogen independently of allosteric activation by glucose-6-phosphate (G6P). Finally, our findings with these mutants points to the presence of a hierarchy of phosphorylation in LGS. In addition, to study the relevance of the phosphorylation of these sites *in vivo*, we are performing overexpression experiments in animal models using recombinant adenoviruses.

Furthermore, we have identified three new phosphorylation sites of LGS, which correspond to residues that do not share homology with those described for the muscle isoenzyme, through the combined use of affinity chromatography and MALDI-TOF mass-spectrometry. We are currently generating a whole battery of reagents to functionally characterise the

phosphorylation of these novel sequences. These tools will allow to determine the functional significance of the phosphorylation of the newly identified sites and the characterisation of their involvement in the pathogenesis of metabolic diseases like diabetes mellitus. In this context, we have the first recombinant adenoviruses encoding for mutants in the novel phosphorylation sites identified by our group, and we are analysing the functional consequences of phosphorylation/dephosphorylation in these sites.

Analysis of the involvement of the phosphorylation/dephosphorylation events in the subcellular localisation of LGS

Among the putative phosphorylatable residues of LGS, we analysed the phosphorylation at site 3a, which corresponds to serine 641 of the rat sequence. This residue is modified by glycogen synthase kinase-3, (GSK3) and its dephosphorylation has been related to the inactivation of LGS. We have analysed the subcellular localisation of LGS when it is phosphorylated in this residue and our results show that the unmodified protein localised to the sites of active glycogen synthesis. These findings indicate that modulation of the activation state of LGS is probably the result of a complex number of phosphorylation/ dephosphorylation events which drive to changes in the subcellular localisation of the enzyme.

Study of the mechanisms of intranuclear localisation of the muscle isoform of glycogen synthase and its functional consequences

Previous data from our group showed that the accumulation of MGS in the nucleus is inversely correlated with the intracellular glycogen levels. The nuclear localisation of this protein is not diffuse, and our first objective was is to first determine its possible association with diverse subnuclear structures. In this context, we have shown that MGS co-localises with PML and p80-coilin, thus indicating that this isoform is potentially associated with Cajal bodies (See Figure 1.). In addition, we have reported that the region comprising amino acids 555-633 is crucial for the concentration and nuclear aggregation of MGS.

We are working on the identification of MGS-interacting proteins when this enzyme is located in the nucleus. The results obtained will provide crucial information as to the subnuclear localisation of this protein as well as its putative nuclear function. The experimental approach involves affinity purification of MGS from nuclei in non-denaturing conditions and the identification of MGS-interacting proteins by mass-spectrometry. We have identified two nuclear MGS-binding proteins that are involved in the regulation of transcription and splicing.

Analysis of the evolution of the glycogen enzymes: functional implications

Our metabolic and evolutionary studies on glycogen metabolism show that the isoenzymes involved arose from a pre-chordate common ancestor by gene duplication. Furthermore, the main isoenzymes involved in the metabolism of glycogen co-evolved in vertebrates in a concerted fashion in order to fulfil the metabolic requirements/functions of the tissues in which they are expressed. Therefore, we propose

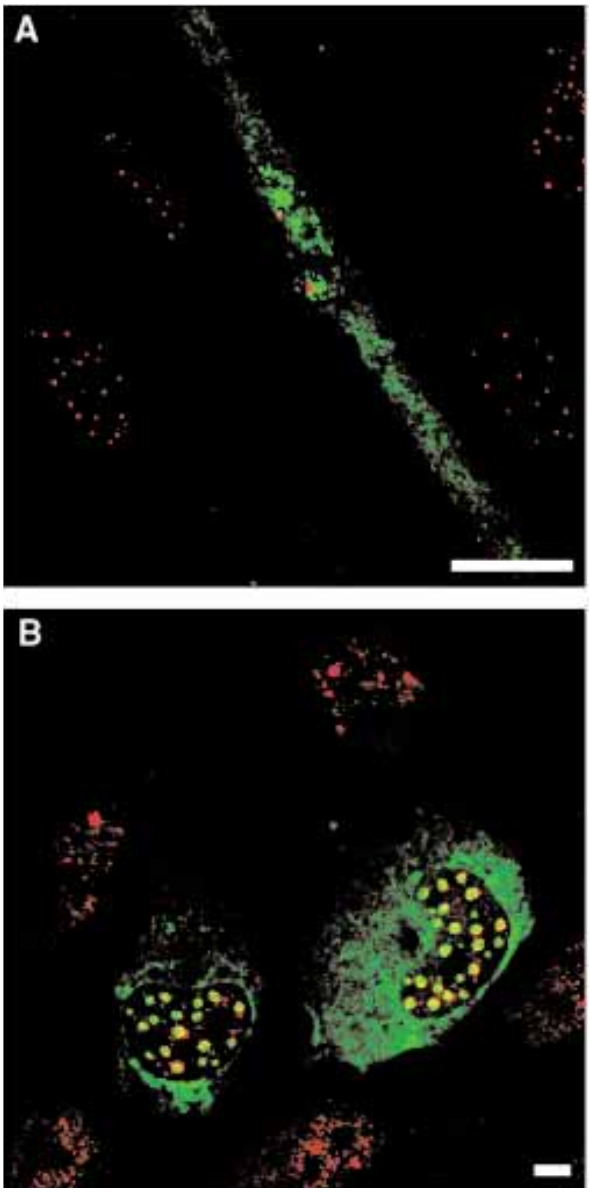

Figure 1*. Co-localisation of HsMGS with markers of subnuclear compartments. (A) Differentiated human myotubes. Cells were subjected to double immunolabelling, to detect MGS (green) and PML (red), respectively. (B) COS-1 cells transiently expressing the GFP-HsMGS fusion protein, in green, immunolabelled with an antibody against p80-coilin, in red. The bar represents 10 µm.*

that tissues are adapted to produce glycogen in their particular metabolic conditions thanks to the concomitant evolution of all the enzymes involved in this process.

The first branch point of glucose metabolism is G6P, whose flux can be diverted either to glycolysis, the pentose phosphate pathway, glycogen synthesis or, in some tissues, back to glucose again. We have shown that the entrance to glycogen metabolism is controlled by the affinity of GS to G6P, rather than physical compartmentalisation of substrates. Moreover, gene duplication allows the independent modulation and evolution of the kinetic features of each GS isoform, thereby allowing liver and muscle to devote glycogen reserves to distinct functions.

Our results show that core metabolic and cellular processes are conserved, but not the mechanisms of control. The addition of control mechanisms enhances the evolvability of the system because they confer flexibility to adapt to new roles. The retroinhibition of high-affinity hexokinases, or GS allosteric activation, both effects mediated by G6P, are clear examples of kinetic features that are modulated through vertebrate evolution to satisfy the diverse roles of glycogen metabolism.

Analysis of the molecular mechanisms involved in the pathogenesis of Lafora Disease

Lafora progressive myoclonus epilepsy (LD, MIM 254780) is an autosomal recessive neurodegenerative disorder characterised by the presence of polyglucosan intracellular inclusion bodies (Lafora bodies), which can be considered aberrant glycogen deposits. Mutations have been identified in two genes, EPM2A (6q24) and EPM2B (6p22.3). The former encodes laforin, a dual-specificity phosphatase with a functional carbohydrate-binding domain. Furthermore, laforin interacts with protein targeting to glycogen (PTG). EPM2B encodes malin, an E3 ubiquitin-ligase that interacts with laforin and promotes its degradation. The physiological roles of laforin and malin are unknown and the cellular processes altered by mutations in these proteins that give rise to the devastating disorder of LD remain to be elucidated. Since Lafora bodies are aberrant molecules of glycogen that accumulate in soma and neural dendrites, we are studying GS activity in neurons and the role of laforin and malin in the regulation of neural glycogen metabolism and, in particular, in the control of GS activity. We have discovered that neurons express MGS (see Figure 2). We are analysing the roles of laforin and malin in the neuronal glycogen metabolism. Our results suggest that laforin has two functions in glycogen synthesis and that it participates in the control of both the activation state and the total levels of key proteins in glycogen synthesis regulation. Taken together, our results indicate that laforin and malin play crucial roles in the maintenance of undetectable or absent levels of glycogen in neuronal cells. In conclusion, laforin and malin are candidates in the control of the intracellular levels of the most important proteins involved in glycogen synthesis, and in preventing the development of glycogen-like intracellular inclusions.

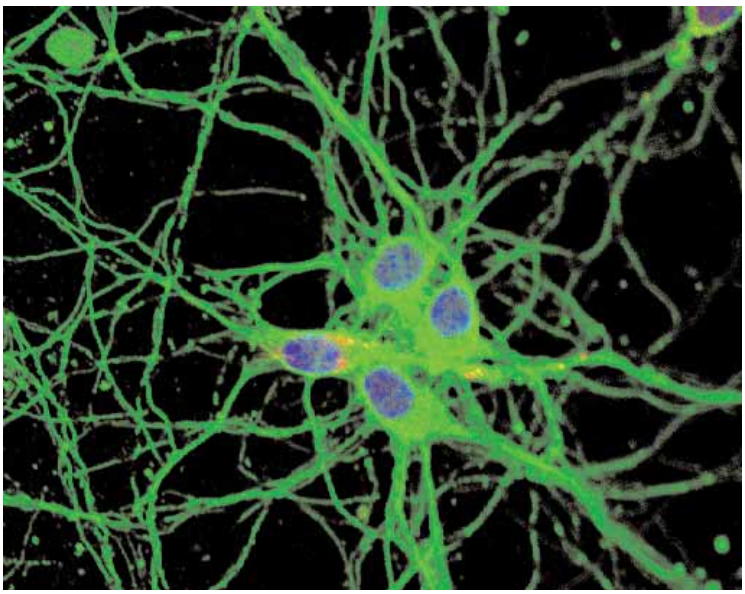

Figure 2. *Immunodetection of MGS in primary cultured neurons. Cells were subjected to double immunolabelling to detect MGS (red) and tubulin (green). Nuclei were stained with Hoechst 33342.*

Analysis of the actions of Lithium ions on glycogen synthase stability

Lithium ions show several remarkable effects on hepatic glycogen metabolism. Thus, LiCl activates LGS in systems such as isolated hepatocytes, primary cultured hepatocytes, and whole animals. This activating action is triggered in the presence and absence of glucose. LGS-induced glycogen synthesis is achieved by both activation and changes in the intracellular location of the enzyme, since effectors that activate GS without causing its translocation fail to stimulate glycogen synthesis. LiCl is a known inhibitor of GSK3 and this property may partly explain the basis of lithium-induced GS activation.

We continued the study of the mechanisms through which LiCl activates LGS and, hence, stimulates hepatic glycogen synthesis. Our results indicate that the LiCl-induced hepatocyte LGS activation is not related to a concomitant translocation of the enzyme. The LiCl action was associated to a significant increase in total GS protein levels with no mod-

ification of GS transcript abundance. Furthermore, the increase in GS levels induced by LiCl was not sensitive to inhibitors of transcription or translation. In contrast, this effect was abrogated by calpain and proteasome inhibitors, suggesting that the mechanism of action of LiCl on the total GS content is related to an increase in the stability of the enzyme.

Dissection of the molecular mechanisms involved in the antidiabetic actions of the oral antidiabetic agent sodium tungstate

Several of our studies describe the anti-diabetic and anti-obesity actions of sodium tungstate when administered orally in animal models of diabetes and obesity. Phase I of clinical trials for this compound has finished and Phase II will start in 2007. Although the metabolic and physiological actions of tungstate have been studied in depth, information on its mechanisms of action is scarce.

Our results show that tungstate exerts its metabolic actions, such as glycogen deposition, amongst others, through a novel mechanism involving the activation of GS and the inactivation of GSK3 through a ras/raf/MEK/ERK/p90RSK dependent-pathway. This process is independent of the insulin receptor, other receptor tyrosine kinases, or the PI3K-PDK1-PKB/Akt transduction pathway branch, the canonical way of inactivating GSK3. The inactivation of this protein kinase contributes to the induction of glycogen deposition stimulated by tungstate. We have used several approaches to attain these results, such as inhibiting several kinases through chemical inhibitors, the use of dominant negative forms of the proteins and expression with adenoviral vectors, among others.

We are currently studying the primary target(s) of tungstate that lead to the downstream events observed: *ie*, activation of ERK and glycogen synthesis deposition. We have preliminary data showing the involvement of PKC and G-proteins in the mechanism of action of tungstate, as well as some data pointing to ionic homeostasis as one of the possible mechanisms modified by treatment with this compound. Furthermore, we have observed inhibition of gluconeogenesis in primary culture hepatocytes when treated with tungstate as compared to untreated cells. Using Real Time PCR and luciferase-coupled assays, we are carrying out several studies on the transcription factors involved in the regulation of the gluconeogenic pathway modified by tungstate.

In addition, we have data indicating that the livers of diabetic animals treated with tungstate show normalisation of the expression of several proteins of the insulin transduction cascade. Moreover, we have also observed a tungstate-dependent normalisation of the state of activation of several components of the insulin cascade, without a modification of total protein amounts.

Our data point to tungstate as an effective anti-diabetic agent and to ERK1/2, PKC and G-proteins as novel targets for the treatment of diabetes.

Crystal structure of glycogen synthase from *Pyrococcus abyssi*: insights into oligomerization and substrate binding of eukaryotic glycogen synthases

The crystal structure of the glycogen synthase from *Pyrococcus abyssi*, the smallest known member of the GS family, revealed that its subunits possess a fold common to other glycosyltransferases a pair of $\beta/\alpha/\beta$ Rossmann fold-type domains with the catalytic site at their interface. Nevertheless, the archaeal enzyme presents an unprecedented homotrimeric molecular arrangement. The C-terminal domains are not involved in intersubunit interactions of the trimeric molecule, thus allowing for movements, likely required for catalysis, across the narrow hinge that connects the N- and C-domains. The radial disposition of the subunits confers on the molecule a distinct triangular shape, clearly visible with negative staining electron microscopy. Comparison of bacterial and eukaryotic glycogen synthases, which use, respectively, ADP or UDP glucose as donor substrates, with the archaeal enzyme, which can utilize both molecules, allowed us to propose the residues that determine glucosyl donor specificity. A structural model of *Pyrococcus abyssi* GS is shown in Figure 3.

***Figure 3**. 3D structure of* Pyrococcus abyssi *GS showing the similarities shared with Human MGS.*

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RESEARCH NETWORKS AND GRANTS

Mechanisms of regulation and signalling in glycogen metabolism. Molecular analysis and its deffects in diabetes
MCYT, BMC2002-00705: 2002-2005
Project Coordinator: Joan J Guinovart

Research on the therapeutic targets of the oral antidiabetic agent sodium tungstate
MEC, SAF2004-06962: 2004-2007
Project Coordinator: Joan J Guinovart

Effects of tungstate on the metabolic syndrome: analysis of plasmatic, hepatic and muscular actions. Determination of therapeutic antions
Insituto de Salud Carlos III, PI042402: 2005 to 2008
Project Coordinator: Joan J Guinovart

Regulation of hepatic, muscular and neuronal glycogen metabolism. Alterations on pathological situations: diabetes mellitus and Lafora disease
MEC, BFU2005-02253/BMC: 2006-2009
Project Coordinator: Joan J Guinovart

OTHER FUNDING SOURCES

Lafora disease: glycogen metabolism and neuronal degeneration
Fundación "La Caixa": 2003-2006
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Novel drugs and targets for the treatment of Diabetes Mellitus
Fundación "Marcelino Botín": 2006-2010
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Study of the antidiabetic actions of tungstate on diabetes induced by immunosupressant treatment
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Laser induced forward transfer: a direct writing technique for biosensors preparation
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Analysis of the toxicity and antidiabetic potential of GSK3 inhibitors
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Joan J Guinovart's group, March 2006.

Primary inherited aminoacidurias and structure-function relationship studies on heteromeric amino acid transporters

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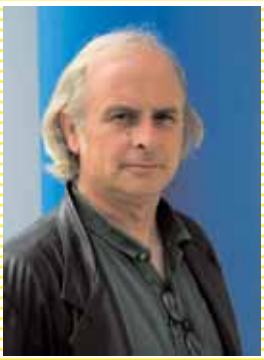

Manuel Palacín

Primary inherited aminoacidurias (PIAs) are rare diseases caused by defective amino acid transport activities, which affect renal reabsorption of amino acids and may also affect intestinal absorption of amino acids and transport function in other organs. Mutations in B0AT1 (*SLC6A19*) cause most cases of Hartnup disorder (Seow *et al*, 2004; Kleta *et al*, 2004). B0AT1 corresponds to system B0 and catalyses the Na^+-dependent transport of most neutral amino acids. Heteromeric Amino Acid Transporters (HATs) are involved in PIAs because mutations in rBAT (*SLC3A1*) and $b^{0,+}$AT (*SLC7A9*) cause cystinuria (Calonge *et al*, 1994; Feliubadalo *et al*, 1999) and mutations in y^+LAT1 cause Lysinuric Protein Intolerance (LPI; Torrents *et al*, 1999).

HATs are composed of a heavy subunit and a light subunit. Two homologous heavy subunits (HSHATs) from the SLC3 family have been cloned and are called rBAT (*ie*, related to b0,+ amino acid transport) and 4F2hc (*ie*, heavy chain of the surface antigen 4F2hc, also named CD98 or fusion regulatory protein 1; FRP1). Ten light subunits (LSHATs; SLC7 family members from SLC7A5 to SLC7A13) have been identified. Six of them are partners of 4F2hc (LAT1, LAT2, y+LAT1, y+LAT2, asc1, and xCT); one forms a heterodimer with rBAT (b0,+AT); and three (asc2, AGT-1 and arpAT) seem to interact with as yet unknown heavy subunits.

Over the last 15 years our group has identified the first members of HATs (Betran *et al*, 1992 a,b), cloned half of the light subunits (Torrents *et al*, 1998; Pineda *et al*, 1999; Feliubadalo *et al*, 1999; Fernandez *et al*, 2005) and reported the role of genes *SLC3A1*, *SLC7A9* and *SLC7A7* in cystinuria Type I, non-I and LPI (Calonge *et al*, 1994; Feliubadalo *et al*, 1999; Torrents *et al*, 1999). At present, we continue to study the pathology of PIA (Chillarón *et al*, 1996; Dello Strologo *et al*, 2002; Font-Llitjos *et al*, 2005) with the aim of identifying new amino acid transporters in order to unravel the molecular bases of renal reabsorption and intestinal absorption of amino acids (Bodoy *et al*, 2005; Ristic *et al*, 2006). One of our main research interests is to decipher the atomic structure and the molecular mechanism of transport of HATs.

HATs show an oligomeric structure

Blue Native gel electrophoresis, cross-linking, and fluorescence resonance energy transfer *in vivo* indicate that system b0,+ is a heterotetramer $[b0,+AT/rBAT]_2$, whereas xCT-4F2hc (system x_c^-) does not stably or efficiently oligomerise. However, substitution of the heavy subunit 4F2hc for rBAT is sufficient to form a heterotetrameric $[xCT/rBAT]_2$ structure. The functional expression of concatamers of two light subunits (which differ only in their sensitivity to inactivation by a sulfhydryl reagent) suggests that a single heterodimer is the functional unit of systems b0,+ and x_c^- (Fernández *et al*, 2006).

The observation that the functional unit of system b0,+ is the heterodimer points to a complex mechanism of amino acid exchange for HATs. The light subunit of these transporters acts as the "catalytic" subunit (true transporter) in the heterodimer, as revealed by reconstitution experiments of b0,+AT (Reig *et al*, 2002). Moreover, it has been shown that system b0,+ from chick small intestine shows a sequential exchange mechanism compatible with the formation of a ternary complex (Torras-Llort *et al*, 2001). If this applies to the expressed b0,+AT-rBAT complex, then export and import pathways should co-exist in the proposed functional unit (*ie*, the heterodimer) and, therefore, in a single b0,+AT catalyt-

ic subunit (Figure 3). To probe this mechanism of transport a deep knowledge of the atomic structure and the structure-function relationships of HATs is needed.

Light subunits

In recent years we have published two studies about the membrane topology (Gasol *et al*, 2004) and relevant residues for transport activity of the HAT light subunit xCT (LAT family of transporters - L-system amino acid transporter; Jimenez-Vidal *et al*, 2004). In the first study, and based on the accessibility of single cysteines to 3-(N-maleimidylpropionyl) biocytin, we proposed a topological model for xCT of 12 transmembrane domains with the N and C termini located inside the cell. The location of these termini was confirmed by immunofluorescence. Studies of biotinylation and accessibility to sulfhydryl reagents revealed a re-entrant loop within intracellular loops 2 and 3. Residues His110 and Thr112, facing outside, are located at the apex of the re-entrant loop. Biotinylation of H110C was blocked by xCT substrates, by the nontransportable inhibitor (S)-4-carboxyphenylglycine, and by the impermeable reagent (2-sulfonatoethyl) methanethiosulfonate, which produced inactivation of H110C that was protected by L-glutamate and L-cysteine with an IC50 similar to the Km. Protection was independent of temperature. Our data indicate that His110 lies close to the substrate binding/permeation pathway of xCT. The membrane topology of xCT could serve as a model for other light subunits of HATs (Figure 1).

In the second study we measured sensitivity to thiol modification of the heteromeric glutamate/cystine transporter 4F2hc/xCT expressed in *Xenopus* oocytes. p-Chloromercuribenzoate (pCMB) and p-chloromercuribenzenesulfonate (pCMBS) rapidly blocked transport activity. Cys327, located in the middle of the eighth transmembrane domain of the light subunit (xCT), was the main target of inactivation. Cysteine, an impermeant reducing reagent, reversed the effects of pCMB and pCMBS only when applied from the extracellular medium. L-Glutamate and L-cystine, but not L-arginine, protected against inactivation, with an IC50 similar to the Km. Protection was not temperature-dependent, suggesting that it did not depend on large substrate-induced conformational changes. Mutation of Cys327 to Ala and Ser slightly modified the Km and a C327L mutant abolished transport function without compromising transporter expression at the plasma membrane. These results indicate that Cys327 is a functionally important residue accessible to the aqueous extracellular environment and that it is structurally linked to the permeation pathway and/or the substrate binding site.

To further increase our knowledge of the structure-function relationship of the light subunits of HATs, we initiated a line of research on the prokaryotic homologues of these subunits. We have identified an orphan protein from *Bacillus sp* as a new member of the LAT family of amino acid transporters. This protein shares an amino acid sequence identity of ~30% with the human light subunits of HATs. The purified protein from *E. coli* membranes was reconstituted in proteoliposomes and showed sodium-independent obligatory exchange activity of L-serine and L-threonine. Kinetic analysis supports a sequential mechanism of exchange characteristics of HAT transporters. Freeze-fracture analysis of the purified and active transporter in proteoliposomes, and Blue Native-polyacrylamide gel electrophoresis and transmission electron microscopy (TEM) of detergent-solubilised purified transporter indicate that the transporter exists in a monomeric form. To our knowledge, this is the first functional characterisation of a prokaryotic member of the LAT family and the first structural data about a transporter of the APC superfamily. This new transporter represents an excellent model to study the molecular architecture of light subunits of HATs and other APC transporters. Currently this and other homologous prokaryotic transporters of the

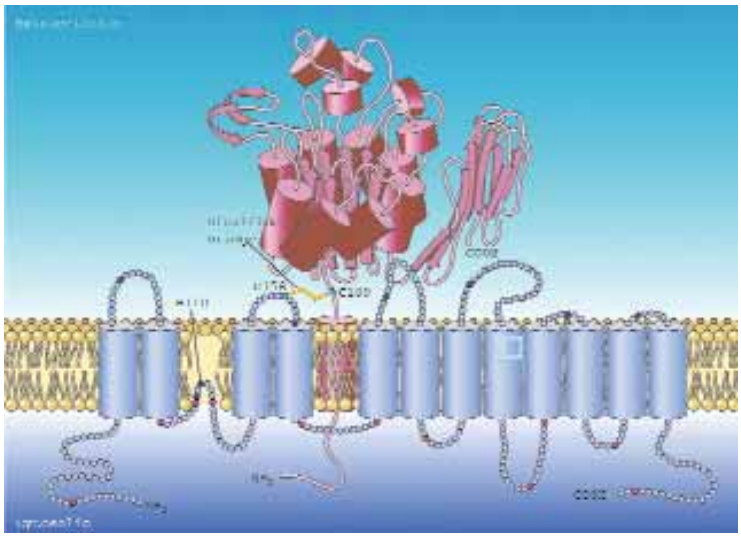

***Figure 1**. A heteromeric amino acid transporter. The heavy subunit (pink) and the light subunit (blue) are linked by a disulfide bridge (yellow) with conserved cysteine residues (cysteine 158 for the human xCT and cysteine 109 for human 4F2hc). The heavy subunits (4F2hc or rBAT) are type II membrane glycoproteins with an intracellular NH2 terminus, a single transmembrane domain, and a bulky COOH terminal. This part of the protein shows homology with bacterial glycosidases. The membrane topology of the light subunit xCT, as model of the other light subunit, shows 12 transmembrane domains, with the NH2 and COOH terminals located intracellularly and with a re-entrant loop-like structure in the intracellular loop IL2-3 (His110 corresponds to the apex of the loop; Gasol* et al*, 2004). Residues with external (black) or internal (red) accessibility are shown. Taken from Palacín* et al*, 2005.*

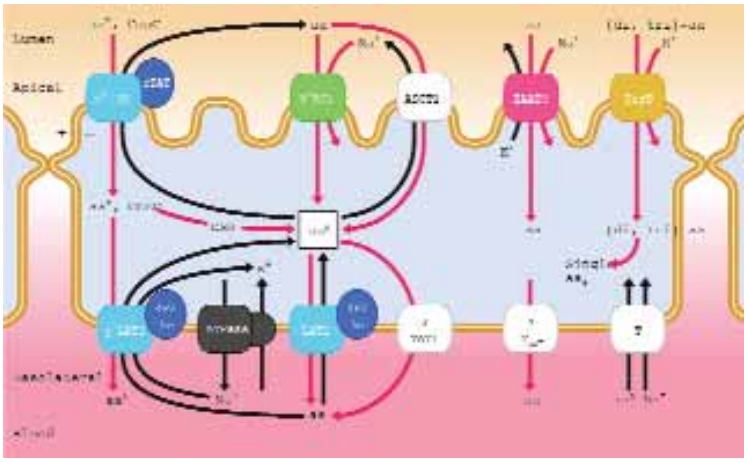

Figure 2. Transporters involved in the renal and intestinal reabsorption of amino acids. Transporters with proven roles in renal reabsorption or intestinal absorption of amino acids are shown in colour. Transporters present in the apical or basolateral plasma membrane of the epithelial cells of the proximal convoluted tubule or of the small intestine, but with no direct experimental evidence supporting their role in reabsorption, are shown in black and white. Fluxes of amino acids in the reabsorption direction are in red. PepT (H+-dependent peptide transporter PepT1 in small intestine and Pept2 in kidney) cotransports di- and tripeptides [(di,tri)-aa] with protons. Intracellular hydrolysis then renders single amino acids. The high intracellular concentration of neutral amino acids (aa0) due to the transport activity of apical (B0AT1) and basolateral (y+LAT1-4F2hc, T) transporters drives the active reabsorption of dibasic amino acids (aa+) and cystine (CssC), together with the membrane potential and reduction of CssC to cysteine (CSH). Basolateral efflux transporters for dicarboxylic amino acids are unknown (Taa-). T represents basolateral Na+-dependent transporters with undefined roles in reabsoption (systems A and ASC). Our group made a key contribution to the determination of the role of transporters rBAT/b0,+AT, 4F2hc/y+LAT1 and 4Fhc/LAT2 in renal reabsorption of amino acids. Taken from Palacín et al, 2005.

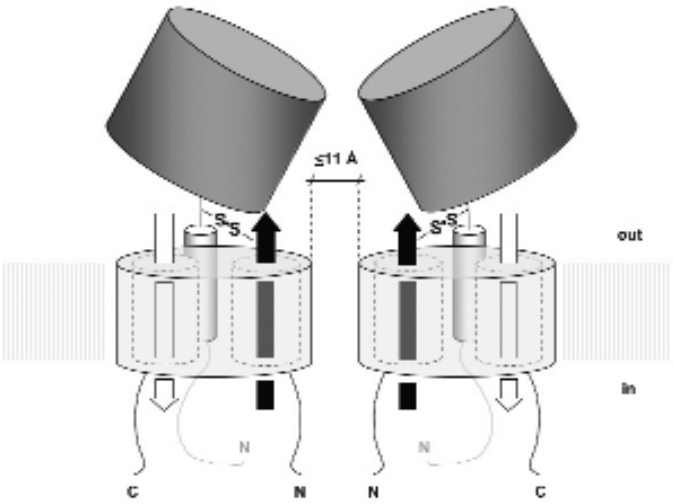

Figure 3. Heterotetrameric structure of system b0,+. Cross-linking experiments revealed that lysine residues in each light subunit b0,+AT are separated by no more than 11 Å. Functional studies revealed that the heterodimer (composed of rBAT and b0,+AT; the light wide cylinder) is the functional unit of the transporter (Fernández et al, 2006). In this functional model, two translocation pathways (one for influx and the other for efflux is present in each light ("catalytic") subunit (see text for details). -S-S-, disulfide bridge connecting rBAT and b0,+AT. In, cytosolic. Out, extracellular.

light subunits of HATs are being studied using 2D and 3D crystallisation.

Heavy subunits

Little is known about the structure of the heavy subunits of HATs (rBAT and 4F2hc). They have a molecular mass of ~90 and ~80 kDa for rBAT and 4F2hc, respectively, and are type II membrane N-glycoproteins with an extracellular COOH terminus (ectodomain) homologous to insect and bacterial glucosidases (Figure 1). Recently, we have solved the atomic structure of the human 4F2hc ectodomain at 2.1 Å resolution. This domain has a similar architecture to bacterial glucosidases with a triose phosphate isomerase (TIM) barrel [$(\alpha\beta)8$] and eight anti-parallel β-strands. Several features of this structure provide insight into the position of the ectodomain on the plasma membrane, potential interaction surfaces with the accompanying light subunits and motifs that might be involved in the multiple functions of 4F2hc in cell fusion, adhesion and transformation. Currently mutants of the 4F2hc ectodomain are being examined to identify the residues involved in the multiple functions of the protein.

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RESEARCH NETWORKS OR GRANTS

Heteromeric amino acid transporters: structure, functional genomics and pathophysiology
MCYT, Plan Nacional, SAF2003-08940-C02-01: 2003-2006
Project Coordinator: Manuel Palacín

European Genomic Initiative on Disorders of Plasma Membrane Amino acid Transporters (EUGINDAT)
European Commission, LSH2002-2.1.1-7
Contract 502852: 2004-2007
Project Coordinator: Manuel Palacín

Inherited metabolic diseases. Network REDEMETH
ISCIII PI050974: 2004-2006
Principal Investigator: Manuel Palacín

Role of 4F2hc in tumourigenesis
La Marató TV3: 2006-2008
Project Coordinator: Manuel Palacín

Heteromeric amino acid transporters: Structure, physiology and pathology
MCYT, Plan Nacional SAF2006-14600-C02-01: 2007-2009
Project Coordinator: Manuel Palacín

Molecular bases of megalencephalic leukoencephalopathy. Biochemical and electrophisicological study
FISS PI04/1680: 2005-2008
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OTHER FUNDING SOURCES

Development of new drugs for cystinuria
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Arginine transport in macrophages
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Development of cystinuria drugs
Laboratories Rubió, Spain

Development of PIA animal models
Ingenium Pharmaceuticals AG, Germany

Metabolic syndrome
Genmedica Therapeutics, Spain

Molecular interactions with 4F2hc
Cristax, Spain

Manuel Palacín's group, March 2006.

Novel mechanisms in the development of insulin resistance and new therapeutic strategies for diabetes

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It has been estimated that between 200 million and 300 million people worldwide will meet World Health Organization diagnostic criteria for diabetes mellitus by the end of this decade. This epidemic of predominantly type 2 diabetes has been mediated largely by our shift toward a more sedentary lifestyle, which predisposes us to obesity and insulin resistance. Individuals affected by this disease may also exhibit an array of associated undesirable effects such as hypertension, dyslipidemia, and hypercoagulability, which lead to morbidity and mortality from atherosclerotic vascular disease. The co-existence of several of these disorders with insulin resistance constitutes the metabolic syndrome. In Western society, metabolic syndrome diseases are growing at epidemic rates and currently affect about 20% of the general population and over 40% of people over 60 years of age. Recent epidemiological and biological data indicate that the etiology of these diseases may share unexpected and common genetic and biochemical mechanisms. A major step towards understanding the metabolic syndrome is the identification of susceptibility genes, which may lead to the acquisition of additional therapeutic targets for future drug design.

In this regard, we study the molecular mechanisms involved in the development of insulin resistance and, in particular, seek to identify novel susceptibility genes for obesity and type 2 diabetes. Our research is structured in three distinct fields: 1) Identification of genes responsible for the development of insulin resistance associated with obesity or type 2 diabetes, with special emphasis on genes implicated in novel mechanisms involving mitochondrial processes (*ie*, mitochondrial dynamics), encoding proteins involved in the regulation of nuclear gene expression, or encoding proteins involved in novel signalling events. 2) Analysis of the molecular mechanisms involved in the regulation of glucose transport in muscle and adipose cells and in the development of lipotoxicity. 3) Identification of novel targets and development of new compounds for the treatment of the metabolic syndrome.

Mfn2 regulates mitochondrial metabolism in non-muscle and muscle cells, is down-regulated in obesity and type 2 diabetes and is induced by PGC-1α

In many cell types, especially muscle fibers, mitochondria form tubular structures or networks (Figure 1). There is evidence in *Saccharomyces*, *Aspergillus* and mammalian cells that mitochondrial filaments are highly dynamic structures and that they are regulated by changes in the rates of mitochondrial fission and fusion. Studies of gain-of-function and loss-of-function have demonstrated that Mfn proteins regulate mitochondrial fusion in mammalian cells. Thus, overexpression of Mfn2 in cultured cells alters mitochondrial morphology, the effects reported ranging from the generation of reticular structures to extensive perinuclear clustering (Bach *et al*, 2003). Mouse embryo fibroblasts lacking Mfn2 or Mfn21 display mitochondrial fragmentation (Chen *et al*, 2003). In addition, Mfn1 and Mfn2 are essential for embryonic development and mice deficient in either gene die in mid-gestation. However, while Mfn2-mutant embryos have a specific and severe disruption of the placental trophoblast giant cell layer, probably caused by a marked reduction in number of placental trophoblastic giant cells, Mfn1-deficient embryos show normal giant cells.

Mfn2 regulates mitochondrial metabolism

Mfn2 is a multifunctional protein and has been reported to inhibit proliferation in smooth muscle cells. Mfn2 also regulates mitochondrial oxidation in

muscle and non-muscle cells. Mfn2 loss-of-function reduces oxygen consumption, mitochondrial membrane potential and oxidation of glucose, pyruvate and fatty acids, without causing changes in mitochondrial mass (Bach *et al*, 2003; Pich *et al*, 2005). In addition, Mfn2 gain-of-function activates glucose oxidation and enhances mitochondrial membrane potential. Overexpression of a truncated mutant of Mfn2 that lacks the transmembrane domains and the C-terminal tail also up-regulates glucose oxidation and increases mitochondrial membrane potential, but has no effect on mitochondrial morphology (Pich *et al*, 2005). These results indicate that Mfn2 participates in mitochondrial oxidation by mechanisms that are independent of its role in mitochondrial fusion.

Regarding the mechanisms responsible for the effects of Mfn2 activity, we have focused on the protein composition of mitochondria. The abundance of mitochondrial protein components of distinct respiratory complexes was assayed in mitochondrial extracts obtained from control and Mfn2 loss-of-function muscle cells. More specifically, we assayed subunit p39 from complex I (encoded by nuclear DNA), protein p70 from complex II (encoded by nuclear DNA), p49 (core 2 subunit encoded by nuclear genome) from complex III, COX-I (encoded by nuclear DNA) and COX-IV subunits of complex IV (encoded by mitochondrial DNA), the β subunit of complex V (encoded by nuclear DNA) and porin (encoded by nuclear DNA). Mfn2 repression caused a marked inhibition of the expression of the subunits of complexes I, II, III and V. In contrast, no alterations in the abundance of complex IV subunits or porin were detected in cells after Mfn2 repression. In keeping with these observations, the enzymatic activity of components I+III and III decreased in Mfn2 loss-of-function cells. These data indicate that Mfn2 loss-of-function causes a specific alteration in the expression of subunits that participate in complexes I, II, III and V, which leads to reduced activity of several components of the OXPHOS system. Mfn2 gain-of-function in L6E9 myoblasts was also associated with increased expression of several subunits of complexes I, IV and V.

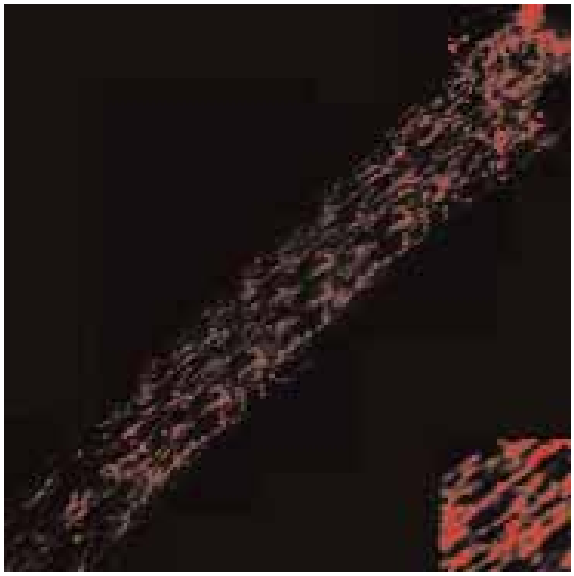

Figure 1. The mitochondrial network in muscle cells. The mitochondrial network was visualized by incubation with a monoclonal antibody against Porin and further incubation with a Texas red-labeled secondary antibody in L6E9 myotubes.

In all, evidence indicates that Mfn2 affects mitochondrial metabolism and its expression level regulates mitochondrial membrane potential, fuel oxidation and the OXPHOS system (Figure 2). Given that a truncated mutant form of Mfn2 that is inactive as a mitochondrial fusion protein maintains its capacity to activate mitochondrial metabolism, we support the view that the metabolic accelerator and the mitochondrial fusion roles of Mfn2 are separate. This hypothesis may explain the exquisite sensitivity of the neuronal cell to a partial Mfn2 loss-of-function, as recently reported in Charcot-Marie-Tooth type 2A neuropathy, a disease that shows autosomic dominant inheritance. On the basis of our data, we propose that Mfn2 loss-of-function participates in triggering the pathological mechanisms that cause disease in highly oxidative tissues such as the nervous system, skeletal muscle or heart.

Mfn2 expression is regulated by obesity and type 2 diabetes

Mitochondrial metabolism is altered in skeletal muscle during insulin-resistant states such as type 2 diabetes or obesity. Human type 2 diabetes is associated with reduced capacity to oxidise glucose in the presence of insulin and, more importantly, to oxidise fatty acid in various conditions. A significant decrease in gene expression of subunits participating in complexes I, II, III and IV of the mitochondrial electron transport chain has also been observed in skeletal muscle of type 2 diabetic patients. Reduced glucose oxidation, decreased pyruvate dehydrogenase activity and enhanced pyruvate dehydrogenase kinase have also been reported in skeletal muscle from diabetic rats. Similarly, experimental diabetes in rats reduces gene expression of various subunits of complexes I, III and IV. In keeping with these observations, the respiratory chain is depressed in skeletal muscle from type 2 diabetic patients and oxidative phosphorylation activity is reduced in heart and skeletal muscle in diabetic rats.

Obesity is also characterised by metabolic alterations which involve mitochondrial defects causing reduced oxidation in skeletal muscle. In some animal models

of obesity such as in obese Zucker rats or ob/ob mice, skeletal muscle shows a metabolic profile characterised by reduced glucose uptake and glucose oxidation, altered partitioning of fatty acids that are incorporated into triglycerides, and reduced oxygen consumption.

In keeping with the regulatory role of Mfn2, we have found that skeletal muscle *Mfn2* expression is reduced in obesity and this affects men and women to a similar extent (Bach *et al*, 2005). In addition, we have observed that *Mfn2* expression in muscle is inversely proportional to the BMI and directly proportional to insulin sensitivity. We have also found that weight loss induced by bilio-pancreatic diversion in morbid obese subjects results in a substantial increase in *Mfn2* expression in skeletal muscle (Bach *et al*, 2005). These data indicate that increased adiposity is linked to repression of Mfn2 mRNA in skeletal muscle and this can be reversed by weight loss. Levels of Mfn2 mRNA strongly correlate with glucose oxidation rates during fasting during euglycemic hyperinsulinemic clamp conditions (Mingrone *et al*, 2005). On the basis of these data, we propose that the increase in Mfn2 mRNA levels explain the increase in glucose oxidation observed in morbid obesity after bariatric surgery.

Our data also indicate that *Mfn2* expression is dysregulated in skeletal muscle from type 2 diabetic patients. In fact, a reduction in *Mfn2* expression is detected in both obese and nonobese type 2 diabetic patients. Low *Mfn2* expression occurs in the presence of reduced expression of the mitochondrial gene COX-III and in the presence of a moderate reduction in citrate synthase mRNA, which suggests the presence of mitochondrial dysfunction.

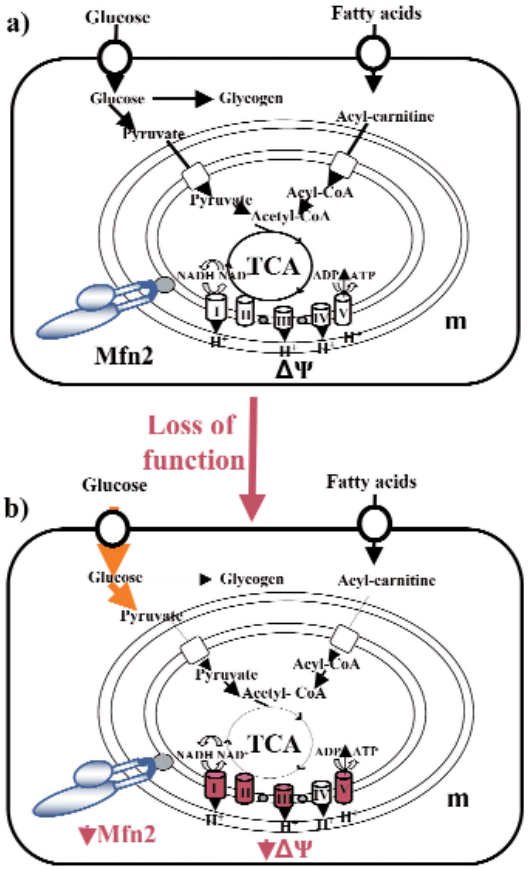

Figure 2. Hypothetical model of the role of Mfn2 in mitochondrial metabolism. a) Cells with a endogenous expression of Mfn2 show normal rates of glucose and fatty acid oxidation and a defined mitochondrial protein composition. b) Mfn2 loss-of-function caused reduced mitochondrial oxidation of glucose and fatty acids, decreased mitochondrial membrane potential and diminished oxidative phosphorylation because of reduced expression of subunits that comprise the respiratory complexes. Under these conditions, glucose uptake is enhanced.

PGC-1α, ERR-α and Mfn2 define a mitochondrial regulatory pathway

Peroxisome proliferator-activated receptor γ (PGC)-1α is a transcriptional co-activator involved in the regulation of genes related to energy metabolism. PGC-1α induces mitochondrial biogenesis and respiration in muscle cells, regulates several aspects of adaptive thermogenesis, gluconeogenesis in liver, and insulin secretion. Overexpression of PGC-1α increases mitochondrial metabolism and this cannot be entirely explained by an increase in mitochondrial mass. In addition, transgenic expression of PGC-1α driven by a muscle specific promoter results in a drastic switch from glycolytic to oxidative fibers. Initially, PGC-1α was described as a tissue-specific co-activator of nuclear receptors but transcription factors of distinct families such as NRF1, MEF2 or FOXO1 are co-activated by this protein. ERRα and GABP are the key transcription factors that regulate the expression of genes of the OXPHOS system mediated by PGC-1α. PGC-1α null mice show, among other defects, reduced mitochondrial function and decreased thermogenic capacity.

We have studied Mfn2 expression under conditions such as exposure to cold or treatment with β_3-adrenergic agonists, which stimulate basal energy expenditure and PGC-1α expression. Exposure to cold for 48 h caused stimulation of Mfn2 mRNA levels in skeletal muscle and in brown adipose tissue. Treatment of rats with the β_3-adrenergic agonist CL-316243 for a range of times also increased *Mfn2* expression in these two tissues.

We have also demonstrated that PGC-1α stimulates *Mfn2* gene expression and that this is due to two elements on the promoter that binds ERRα (Soriano *et*

al, 2006). These data permit us to propose the existence of a regulatory pathway that drives mitochondrial metabolism and is defined by PGC-1α, ERRα and Mfn2 (Figure 3A). The pathway is characterised by a stimulatory action of PGC-1α on the transcription of Mfn2, via co-activation of ERRα. This is supported by the following experimental evidence: a) PGC-1α activates Mfn2 expression in cells; b) the mechanisms by which PGC-1α stimulates this expression are dependent on intact ERRα binding in the Mfn2 promoter; c) Mfn2 regulates mitochondrial metabolism; d) PGC-1α action explains the stimulatory effect of cold-exposure or treatment with the β_3-adrenergic agonist CL-316243 on *Mfn2* expression in muscle and brown adipose tissue.

In addition, we have shown that cold exposure causes an additional increase in PGC-1α in skeletal muscle from mouse knock-outs heterozygous for Mfn2. These results suggest a possible homeostatic Mfn2-induced process that regulates PGC-1α (Figure 3B). This mechanism does not involve a direct effect of Mfn2 as RNAi-induced repression of Mfn2 does not alter PGC-1α expression in C2C12 muscle cells.

Mfn2 is a key target of the nuclear co-activator PGC-1α. Previous studies show that the up-regulation of PGC-1α enhances total mitochondrial membrane potential in cells by increasing mitochondrial number and also by energisation of mitochondria. Mfn2 also enhances mitochondrial membrane potential and several observations support that Mfn2 stimulates mitochondrial proton leak; the effects of Mfn2 are independent of mitochondrial mass. These data support the view that Mfn2 and PGC-1± have common effects on mitochondria. The maintenance of a normal expression of Mfn2 is critical for the stimulatory effect of PGC-1α on mitochondrial membrane potential; in contrast, the effects of PGC-1α on mitochondrial biogenesis are independent of Mfn2. These data suggest that the effects of PGC-1α on mitochondrial energisation require or are mediated by Mfn2. On the basis of the biological roles of Mfn2 reported, we also propose that PGC-1α regulates mitochondrial fusion/fission events and cell proliferation in cells.

Identification of novel substrates of semicarbazide-sensitive amine oxidase/VAP-1 enzyme

Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1) is a bifunctional protein with copper-containing amine oxidase activity (EC 1.4.3.6) that converts primary amines to aldehydes, with the concomitant production of hydrogen peroxide and ammonia. SSAO/VAP-1 is highly expressed in adipocytes where it is localised mainly in plasma membrane in an insulin-independent manner (Enrique-Tarancon *et al*, 1998). Substrates of SSAO/VAP-1 exert a variety of insulin-like effects in human, rat and mouse adipose cells. Thus, substrates such as benzylamine or tyramine stimulate glucose transport in isolated human adipocytes. Furthermore, in isolated rat or 3T3-L1 adipocytes, the combination of SSAO substrates at low ineffective vanadate concentrations causes a potent stimulation of glucose transport, GLUT4 recruitment to the cell surface, lipogenesis and inhibition of lipolysis (Zorzano *et al*, 2003; Marti *et al*, 2004).

In this regard, in collaboration with Fernando Albericio (IRB Barcelona); Miriam Royo (PCB) and Luc Marti (Genmedica Therapeutics), we have identified potent SSAO/VAP-1 substrates based on arylalkylamines. In a first step, structure activity relationships were studied for SSAO/VAP-1 using a library of aryalkylamine substrates. This analysis has revealed the relevance of the electronic properties of the aryl substituents in the activation or inactivation of the SSAO enzymatic process (Yraola *et al*, 2006). The experimental data have been contrasted with the findings obtained in previous homology studies in order to elucidate the mechanism and the substrate-binding affinity of SSAO. In addition, the results have been compared with some recent studies about the Amino Oxidase family and reveals new trends in SSAO substrate design. Thus, we have identified a novel compound, 4-phenylbuthylamine, as a potent substrate for human SSAO, which can be used in the development of anti-diabetic compounds (Yraola *et al*, 2006).

A novel signalling pathway responsible for contraction-induced glucose transport in skeletal muscle

Skeletal muscle is the main tissue responsible for insulin-stimulated glucose utilisation in absorptive states. The rate-limiting step for muscle glucose utilisation is glucose transport, which can be rapidly induced by translocation of GLUT4 glucose transporters from intracellular vesicles to the plasma membrane. Muscle contraction and insulin act independently through distinct signalling pathways to induce GLUT4 translocation. Most interestingly, in insulin-resistant states, such as type 2 diabetes, the effects of contraction on glucose uptake are unchanged. Thus, we have focused our research efforts on studying the molecular mechanisms involved in contraction-stimulated glucose uptake.

Muscle contraction induces glucose transport by a mechanism involving local factors within the myocyte. It has been suggested that two main effectors mediate contraction-induced glucose transport, namely activation of AMP-activated protein kinase (AMPK), a metabolic fuel gauge regulated by cellular

energy charge, and transient increases in cytosolic Ca^{2+} within myofibers, as a consequence of plasma membrane and T-tubule depolarisation.

Neuregulin, a growth factor involved in myogenesis, has rapid effects on muscle metabolism. In a manner analogous to insulin and exercise, neuregulins stimulate glucose transport through recruitment of glucose transporters to surface membranes in skeletal muscle (Suárez *et al*, 2001; Cantó *et al*, 2004). Like muscle contraction, neuregulins have additive effects with insulin on glucose uptake. In collaboration with Anna Gumà and Carles Cantó (University of Barcelona), we have examined whether neuregulins are involved in the mechanism by which muscle contraction regulates glucose transport.

We have found that muscle contraction stimulates the phosphorylation of ErbB4 and ErbB2 but not ErbB3 receptors (Cantó *et al*, 2006). This is consistent with the finding that *in vitro* exposure of skeletal muscle to saturating exogenous neuregulins induces tyrosine phosphorylation of ErbB4 and ErbB2 but induces only weak phosphorylation of ErbB3. These results indicate that ErbB2 and ErbB4 are the main neuregulin receptors in adult skeletal muscle. Caffeine treatment at a concentration that induces Ca^{2+} release to subcontractile levels also mimics contraction effects on ErbB receptor phosphorylation (Cantó *et al*, 2006).

We have also shown that caffeine-induced increases in cytosolic Ca^{2+}-mediate a metalloproteinase-dependent release of neuregulins, which explains the activation of ErbB4 receptors. In addition, activation of ErbB4 is required for Ca^{2+}-derived effects on glucose transport and ErbB4 blockage impairs neuregulin- and caffeine-induced glucose transport in incubated soleus muscle (Cantó *et al*, 2006). In contrast, treatment with specific antibodies that induce ErbB3 blockage do not affect neuregulin- or caffeine-induced glucose transport in soleus muscle.

Blockage of ErbB4 abruptly impairs contraction-induced glucose uptake in slow twitch muscle fibers, and to a lesser extent, in fast twitch muscle fibers. Thus, injection of saturating concentrations of ErbB4 blocking antibody into soleus and EDL muscles before inducing contraction by electrical stimulation of the sciatic nerve inhibited contraction-induced ErbB4 phosphorylation by 86% in soleus and 91% in EDL muscles with a concomitant 71 and 36% impairment in contraction-induced glucose uptake, respectively (Cantó *et al*, 2006). In conclusion, we provide evidence that contraction-induced activation of neuregulin receptors is required for the stimulation of glucose transport and represents a key component of energy metabolism during muscle contraction.

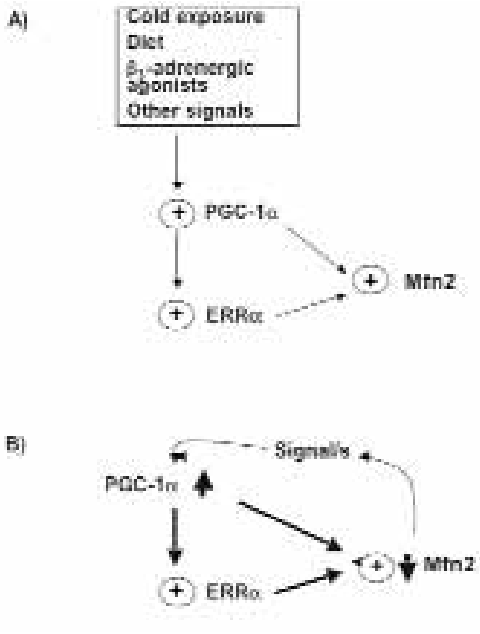

Figure 3. Proposed model for: a) the regulatory pathway defined by PPARγ co-activator 1α (PGC-1α), ERRα and mitofusin-2, and b) feed-back regulation between Mfn2 activity and PGC-1α expression.

Role of mannan-binding lectin in insulin resistance

Substantial evidence has accumulated over the last decade that the metabolic syndrome is linked to inflammatory pathways. The immune system is constantly exposed to diverse bacterial products. The sensing arm of this immune system is efficient in buffering these bacterial and environmental substances, thereby precluding their interaction with membrane-associated receptors of monocytes/macrophages. Mannan- or mannose-binding lectin (MBL) is a liver-derived serum protein involved in innate immune defence. The ligands for MBL are mannose and N-acetyl glucosamine oligosaccharides, expressed by a wide range of microorganisms. MBL may activate complement by means of the lectin pathway when interacting with MBL-associated serine proteases and can directly opsonise pathogens and enhance the activity of phagocytes by means of novel receptors. Interestingly, there is a correlation between increased concentration of certain complement components (C3) and decreased insulin action. Increased levels of C3 and other inflammatory markers in insulin resistance seem to result from chronic inflammation induced by 'excess' fat tissue but it could also be the result of the relative incapacity to buffer the environmental inducers of inflammation. In this regard, decreased inherited capacity to sense environmental substances would lead to increased

exposure of cell immunity to these products and low-grade chronic inflammation.

MBL is an excellent model to test this hypothesis. MBL deficiency is the most frequent immunodeficiency in humans, and leads to an increased incidence of infections in subjects with MBL gene mutations. Serum MBL concentrations vary widely from person to person because of three variant alleles (*B*, *C*, and *D*, denoting the substitution of aspartic acid for glycine at codon 54, the substitution of glutamic acid for glycine at codon 57, and the substitution of cysteine for arginine at codon 52, respectively) in the structural moiety of the functional MBL gene, *MBL2*, located on chromosome 10 in humans. The normal allele of *MBL2* is named *A*, and the common designation for the variant alleles is *O*. Each of the three variant alleles influences the stability of the final protein product, thereby resulting in reduced serum levels and a dysfunctional MBL variant with a lower molecular weight than the normal protein.

In collaboration with José Manuel Fernández-Real and Joan Vendrell, we have studied the levels of circulating MBL levels and the frequency of *MBL2* mutations in obese subjects and the metabolic effects of MBL in muscle. Our results indicate that in non-diabetic men, age-adjusted serum MBL is significantly lower among obese subjects than in lean subjects in parallel to increased serum C-reactive protein, interleukin 6 and the soluble fraction of tumour necrosis factor-α receptor R1 (Fernández-Real *et al*, 2006). At least, one *MBL2* mutation was present in 48% of obese versus 39% of non-obese subjects. In addition, the plasma concentration of MBL-A was lower in insulin resistant obese *ob/ob* mice than in lean mice of the same strain in parallel to decreased glucose/insulin ratio and incubation of rat soleus muscle with human MBL markedly increased fatty acid oxidation (Fernández-Real *et al*, 2006). These findings indicate that MBL, previously thought to function only in inflammation and the immune system, affects metabolic pathways and possibly influences the development of insulin resistance and obesity.

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Zorzano A and Pich S (2006) What is the biological significance of the two mitofusin proteins present in the outer mitochondrial membrane of mammalian cells? IUBMB Life, 58:441-443

RESEARCH NETWORKS AND GRANTS

Mitofusin-2 gene and risk of obesity or type 2 diabetes
Instituto de Salud Carlos III: 2005-2007
Project Coordinator: Antonio Zorzano

Molecular pathologies associated to defective activity of membrane transporters

DURSI, Generalitat de Catalunya: 2005-2007
Project Coordinator: Antonio Zorzano

Functional analysis of insulin resistance candidate genes
Plan Nacional de Biomedicina: 2005-2008
Project Coordinator: Antonio Zorzano

Functional and proteomic characterisation of GLUT4 endocytosis in adipocytes
Plan Nacional de Biomedicina: 2004-2007
Project Coordinator: Marta Camps

Role of caveolae in the physiology of adipose tissue
Ministerio de Educación y Ciencia: 2006-2007
Project Coordinator: Marta Camps

OTHER FUNDING SOURCES
Development of compounds with relevant activities in adipose tissue
Provital SA: 2006
Project Coordinator: Antonio Zorzano

COLLABORATIONS
Functional role of SSAO
Christian Carpéné (INSERM U586, Toulouse, France)

Functional role of Mfn2
David Chan (California Institute of Technology, Pasadena, USA)

Identification of type 2 diabetes susceptibility genes
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Mark McCarthy (Oxford Diabetes Centre, Oxford, UK)
Geltrude Mingrone (Università Cattolica, Rome, Italy)
Federico Soriguer (Hospital Carlos Haya, Málaga, Spain)
Joan Vendrell (Hospital Joan XXIII, Tarragona, Spain)
Hubert Vidal (INSERM U-449, Lyon, France)
Juleen R Zierath (Karolinska Hospital, Stockholm, Sweden)

Identification of SSAO substrates
Luc Marti (Genmedica Therapeutics, Barcelona, Spain)

Synthesis of novel SSAO substrates
Miriam Royo (Combinatorial Chemistry Unit, Parc Cientific de Barcelona, Spain)

Synthesis of novel antidiabetic compounds
Fernando Albericio (IRB Barcelona, Spain)

Hepatic function of Mfn2
Joan Guinovart (IRB Barcelona, Spain)

Antonio Zorzano's group, March 2006.

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Eleonora Marcucci (Italy)

Fernando Albericio

Our research activities are devoted to the discovery and synthesis of bioactive compounds with a focus on the therapeutic fields of cancer and inflammation. We adopt an integrated approach, based on a strong methodological platform and solid-phase and combinatorial science techniques, to synthesise peptides and small molecules. Joint collaborations with biologists and researchers from industrial sectors afford us a unique position in the field.

Methodology
We are continuously developing new methods to prepare innovative scaffolds, building blocks in solution and in solid-phase as well as new tools such as resins, protecting groups, and analytical methods to support the techniques developed.

HFA chemistry
We have developed and adapted the chemistry of hexafluoroacetone (HFA), which is a bidentate protecting/activating reagent for ±-functionalised carboxylic acids, to a solid-phase scheme. The lactones formed from ±-hydroxy acids represent active esters, which, on nucleophilic attack, yield carboxylic acid derivatives. Concomitantly, the ±-functionality is unblocked. Furthermore, we have used HFA derivatives to prepare non-natural amino acid derivatives, which have been incorporated into peptides that show biological activity of interest (Albericio *et al*, 2005). This chemistry has been used in the preparation of twisted and siamese peptides, new topological peptides as well as for chimera peptides (Cupido *et al*, 2005; see Figure 1.)

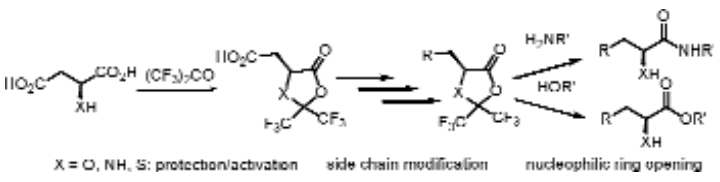

Figure 1. *Hexafluoroacetone as a bidentate protecting/activating reagent for α-functionalized carboxylic acids.*

pNZ as orthogonal protecting group p-Nitrobenzyloxycarbonyl (pNZ) has been used as a temporary protecting group for ±-amino functionalities in SPPS. The pNZ moiety is orthogonal with the most common protecting groups used in peptide chemistry, and is removed under neutral conditions in the presence of catalytic amounts of acid. The use of pNZ derivatives in conjunction with Fmoc chemistry circumvents typical side-reactions associated with the use of piperidine. We have used this technology to develop a convergent synthesis of the anti-tumoural peptide Kahalalide F (clinical phase II; Llobet *et al*, 2005) as well as other bioactive peptides. (See Figure 2.)

ChemMatrix resin
This totally PEG-based resin, made exclusively from primary ether bonds and therefore highly chemically stable, exhibits good loading and is user friendly because of its free flowing form upon drying. It shows excellent performace in the preparation of hydrophobic, highly-structured, and poly-Arg peptides compared to polystyrene (PS) resins. In the most striking example, stepwise solid-phase assembly of the highly complex ≤ amyloid (1-42) peptide resulted in a crude

material of 91% purity. In contrast, procedures described in the literature and using PS- or PEG-PS-based resins for this peptide require convergent approaches and/or additional time-consuming steps.

Anti-tumoural compounds

Small molecules bind to DNA through several mechanisms: (i) minor groove binding; (ii) major groove binding; and (iii) intercalation. The capacity of planar polyheterocyclic aromatic molecules to intercalate, *ie*, to be inserted between two consecutive base pairs of DNA, is of special relevance since many intercalators are active in anti-tumour chemotherapy. The strength of binding usually correlates with the biological activity shown by the molecule, and several energy contributions may be responsible for the binding. All intercalators bind to DNA by non-covalent stacking with nucleic acid base pairs, often combined with H-bonding and even covalent binding involving the drug side chains. Because most intercalators are highly polar or even charged systems, it is believed that electrostatic energy plays a dominant role in intercalation, at least in sequence preferences and drug positioning.

During the last 30 years, many small molecules with a nitrogen-containing polyheterocyclic structure have been isolated from marine invertebrates. These compounds show unique chemical structures that have no precedents in natural products from the plant kingdom or from synthetic approximations, and their cytotoxicity in a number of tumour cell lines made them good candidates to develop new chemotherapeutic compounds against cancer. Several authors postulate that the interaction with DNA is responsible for the cytotoxicity of these marine-derived compounds. These natural compounds open up two areas for further investigation:

- The development of novel synthetic strategies for the preparation of unprecedented structures in larger amounts than those found in natural sources.
- The improvement of the activity shown by these structures.

Lamellarins

We have developed efficient modular synthetic routes to open chain alkaloids related to Lam-D. The synthetic strategy is based on the preparation of a 5,6-dihydropyrrolo[2,1-b]-isoquinoline scaffold from a methyl pyrrole-2-carboxylate, and protocols enabling regioselective bromination followed by Suzuki cross-coupling have been established for the introduction of aryl groups onto the 2- and 3-positions. (Olsen *et al*, 2005). We have demonstrated the utility of this procedure for the preparation of analogs of Lam-D with one aryl-group, with two equivalents or distinct aryl groups and also for the rapid synthesis of the natural product, Lam-D. (See Figure 3.)

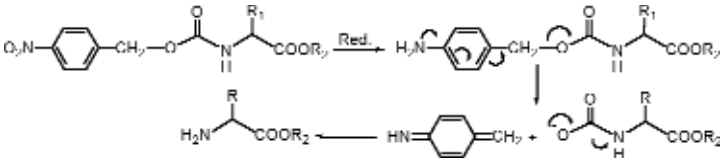

***Figure 2**. Mechanism of pNZ removal.*

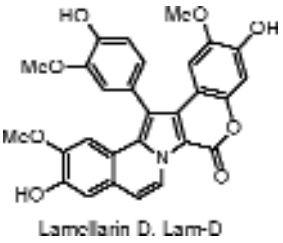

***Figure 3**. Structure of lamellarin D.*

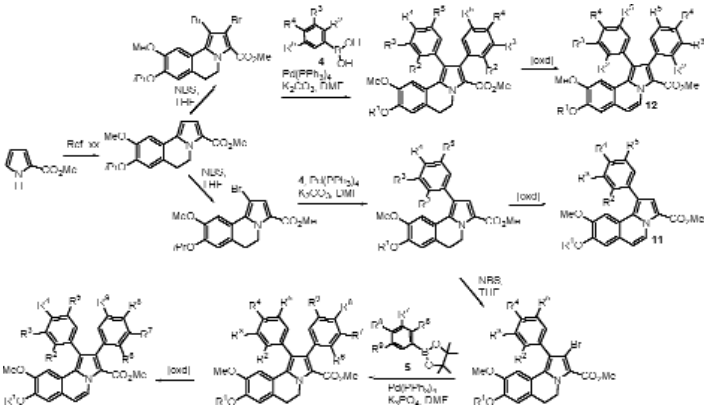

***Figure 4**. Synthesis of library of open chain lamellarins.*

Library of lamellarin

Lam-D is one of the most potent lead candidates for anti-cancer chemotherapy. We have prepared a small library of open chain lamellarin analogues using the previously described (Olsen *et al*, 2005) methodology in solution (Pla *et al*, 2006). The synthesis starts from methyl pyrrole-2-carboxylate by transformation into

the scaffold from which the monoaryl- and diaryl-analogues were prepared. The regioselective bromination of the scaffold followed by a Pd(0)-catalysed Suzuki cross-coupling reaction, oxidation and deprotection was the synthetic strategy followed for the total synthesis of Lam-D. The isopropyl ether gave good yields as protecting groups for the phenols for

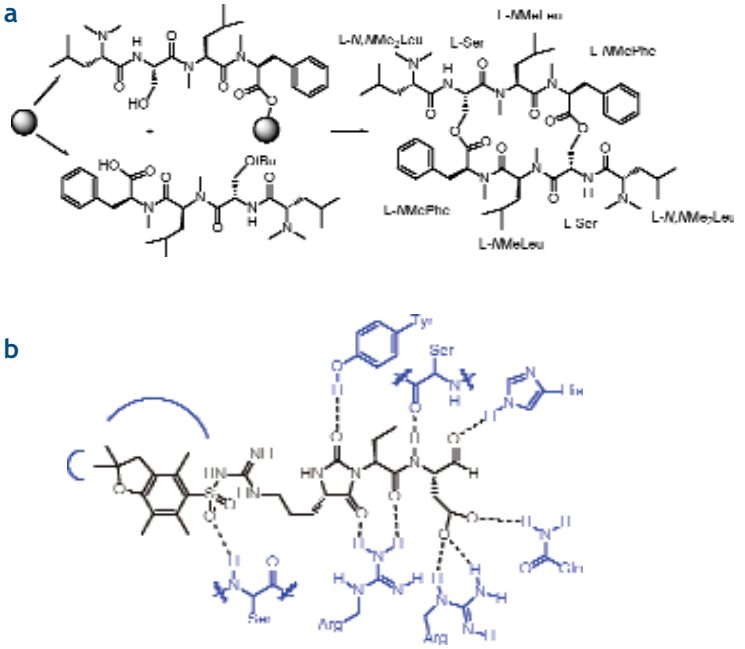

***Figure 5**. Interactions by molecular dynamics.*

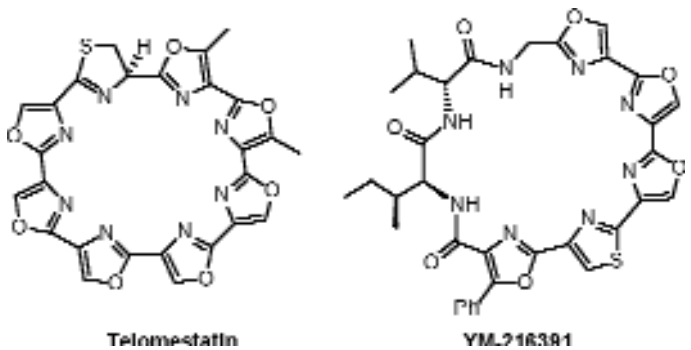

***Figure 6**. Telomestatin and YM-216391.*

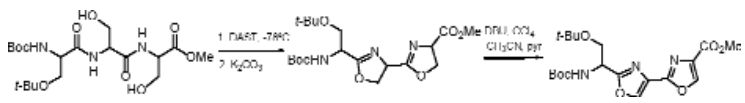

***Figure 7**. Synthesis of concatenated azoles.*

and was therefore used in the present synthetic process. In these compounds, diversity is introduced with the aryl groups. For this purpose, we use commercial arylboronic acids or borolanes prepared in our laboratory. The key step in the synthetic process is the introduction of the aryl substituents on positions 1 and 2 of the scaffold using the boron derivatives as a building blocks for the construction of the final structure. (See Figure 4.) Following this methodology, the synthesis of IB-01211 (see Figure 10) is in course in our laboratory. IB-01211 is a natural product isolated from a marine-derived microorganism strain ES7-088. This new cyclopeptide presents strong cytotoxic activity against several tumour cell lines. The application of the synthetic methodology to the preparation of modified compounds to improve the solubility or the pharmacological profile is also in process.

Concatenated azoles as telomerase inhibitors

The finite lifespan of somatic cells results from the shortening of single-stranded "telomeric" DNA protecting the ends of the chromosomes. These telomeres are progressively shortened with successive rounds of replication until they reach a critical length at which apoptosis is triggered to prevent further aging. However, tumour cells can maintain their telomeric ends by the action of telomerase. This enzyme has reverse-transcriptase properties and is overexpressed in >85% of all malignant cells but is virtually absent in healthy tissue (Kim *et al*,1999). It therefore, constitutes a particularly promising molecular target in the quest for more selective chemotherapeutic agents against cancer (Neidle *et al*, 2005).

Telomestatine, which was isolated from *Streptomyces anulatus* 3533-SV4 (Shin-ya *et al*, 2001) and is an example of 1,4-polyazole condensed compounds, interacts specifically with the human telomeric intramolecular G-quadruplex without affecting DNA polymerases and reverse transcriptases. In this addressed the preparation of catenated azoles as telomerase inhibitors.

The use of peptide derivatives (from serine or threonine) is the most common approach for the synthesis of polyoxazole fragments by iterative cyclodehydration-oxidation steps (Riego *et al*, 2005). We have prepared bisoxazole subunits by simultaneous formation of the two heterocyclic rings in moderate yield. The chemical procedure is based on the treatment of serine peptides with DAST and a base, followed by oxidation with DBU in CCl4, CH3CN, and pyridine. (See Figure 7.)

This protocol allows the preparation of more complex products such as trisazoles, tetraoxazoles, and pen-

taoxazoles when R1 or/and R2 are azole rings, as shown in Figure 8.

Moreover, we have synthesised a new cyclic trisoxazole-peptide hybrid by reacting the corresponding acid of the trisoxazole and the tripeptide H-β-Ala-Ile-Val-OMe, followed by deprotection of the acid and amine functions, and cyclisation with DIPCDI/HOAt. (See Figure 9.)

The compounds with a proper pharmacological profile may also require special treatment for clinical administration because of their low solubility.

In a similar context, we are currently preparing building blocks (amino acids) bearing diversely functionalised heterocyclic units [(iso)oxazole and (iso)thiazole] to develop flexible synthetic schemes for natural product precursors.

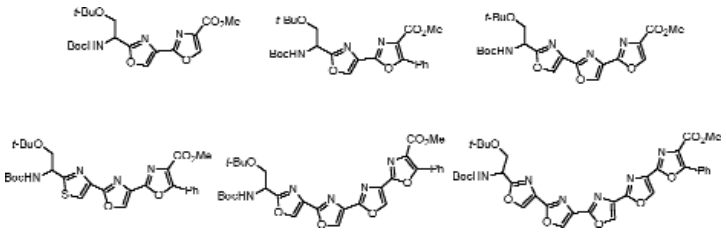

Figure 8. Structures of synthesised polyazoles.

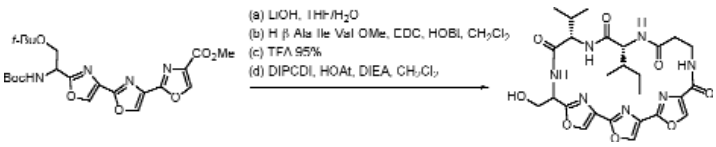

Figure 9. Cyclic peptide containing a trioxazole.

Cyclic peptides of marine origin

In addition to synthesis of new analogues of Kahalalide F (currently in clinical phase II: Albericio *et al*, PCT Int Appl 2005), the total solid-phase synthesis of the cyclodepsipeptide IB-01212 has been performed in parallel via three distinct routes: dimerisation of heterodetic fragments, linear synthesis, and convergent synthesis, the latter yielding the best results (Cruz *et al*, 2006). We used these strategies, in combination with orthogonal protecting groups, to prepare analogues of IB-01212 and other peptides with a C2 symmetry. (See Figure 5a.)

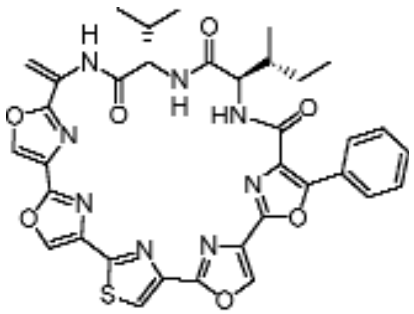

Figure 10. Structure of IB-01211.

Oxathiocoraline

Thiocoraline is one of several new potent antitumoural agents that have been isolated from the marine organism the *Micromonospora sp*. (Romero *et al*, 1997) Thiocoraline inhibits DNA elongation by DNA polymerase ± at a concentration that blocks cell cycle progression and clonogenicity (Erba *et al*, 1999). However, a main drawback for the clinical use of this agent is its low solubility in all media used for delivery. To overcome this drawback, an alternative is the preparation of compounds with a close topology, but with a different solubility pattern. Thus, the oxa derivative of Thiocoraline, where the thioester bonds have been substituted by ester bonds (from the building block perspective implies the use of Ser residues instead of Cys) has been obtained. (See Figure 11.)

The synthetic scheme is based on a three-orthogonal scheme (*t*-butyl, fluorenylmethyl, allyl) with trityl resin. A cornerstone of our approach is the coupling reagents used. Thus, aminium and phosphonium salts are used in solid-phase, thereby facilitating the removal of excess and side-products by filtration and washing. While HATU is used for the step-wise elongation of the peptidic chain to assure a complete acylation of N-methylamino acids; PyAOP is preferred for fragment condensation because it does not provoke capping of the amino function in slow couplings. For solid-phase cyclisation, HOAt, as additive of DIPCDI, gives better results than HOBt. Cyclisation and incorporation of 3-hydroxyquinaldic acid is performed in solution with EDC·HCl to allow easy removal of carbodiimide derivatives during the work-up. While HOAt is the best additive for the cyclisation, the less reactive HOSu is preferred for the final acylation, because it prevents over-incorporation of the carboxylic acid.

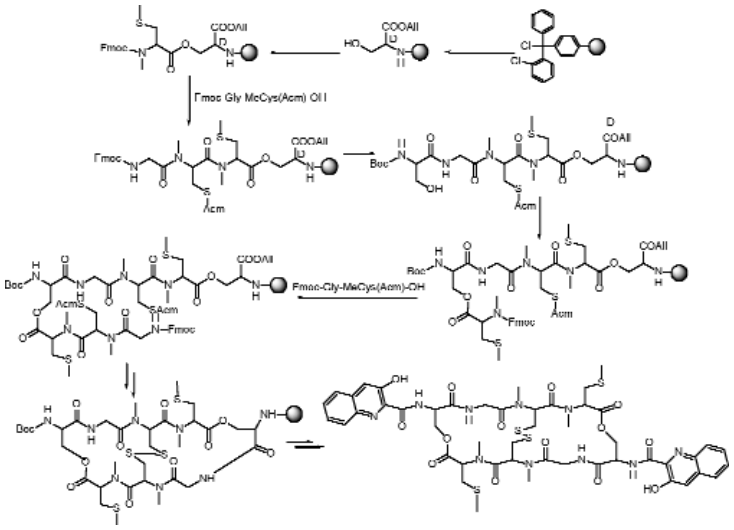

Figure 11. Synthesis of oxathiocoraline.

Peptidomimetic libraries: azabicyclo-δγ-bilactam as a new scaffold for caspase-3 inhibitors

Hydantoins have been used as a new scaffold to synthesise a new kind of peptidomimetic caspase-3 inhibitor, introducing a basic group in the S_3 subsite . This will reduce the overall negative charge of caspase-3 inhibitors improving their cell permeability . Figure 5b shows the interactions of the most potent analogue with caspase-3 as studied by molecular dynamics. New libraries have been prepared taking as a base the promising results obtained in the previous project (results pending publication).

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RESEARCH NETWORKS AND GRANTS

Combiestrategias para el descubrimiento de nuevos fármacos peptídicos y/o heterocicliclos
Comisión Interministerial de Ciencia y Tecnología (BQU2003-00089): 2004-2006
Principal Investigator: Fernando Albericio

Disseny i síntesi de pèptis i peptidomimètics neutralitzants d'endotoxines bacterianes com agents terapèutics and capacitat antiinflamatòria
Fundació La Marató de TV3: 2004-2006
Principal Investigator: Fernando Albericio

Síntesis racional de moléculas con afinidad al ADN de doble cadena constitudos por diversas unidades activas por el mecanismo de intercalación
Comisión Interministerial de Ciencia y Tecnología (PETRI 95-0976): 2005-2007
Principal Investigator: Fernando Albericio

Purificación de Proteínas de interés farmacéutico
Colaboración con la Universidad de Buenos Aires, Argentina - A/3995/05
Secretaría de Estado de Cooperación Internacional (AECI) - Ayudas para proyectos conjuntos de investigación: 2006
Principal Investigator: Fernando Albericio

Nanobiotecnología para la terapia y el diagnóstico de tumores sólidos cancerígenos
Colaboración con la Universidad de Santiago, Chile - A/3116/05
Secretaría de Estado de Cooperación Internacional (AECI) - Ayudas para proyectos conjuntos de investigación: 2006
Principal Investigator: Fernando Albericio

Plataforma combiquímica basada en productos naturales: descubrimiento y administración de fármacos
Ministerio de Educación y Ciencia: 2006-2008
Principal Investigator: Fernando Albericio

OTHER FUNDING SOURCES
Nanobiotecnología basada en productos marinos para la terapia de tumores sólidos cancerígenos
CENIT-Nanopharma with Pharmamar SA: 2006-2007
Principal Investigator: Fernando Albericio
Produgs y bioconjugados
CENIT-Nanopharma with Pharmamar SA: 2006-2007
Principal Investigator: Fernando Albericio

Apoyo tecnológico referente a la preparación y desarrollo de librerías de compuestos de química combinatoria
CENIT-Nanopharma with Almirall Prodesfarma: 2006-2007
Principal Investigator: Fernando Albericio

Promotores de absorción ocular
CENIT-Nanopharma with Sylent SA: 2006-2007
Principal Investigator: Fernando Albericio

Consortium: Centros de Investigación Biomédica en Red (CIBER) - Bioingenierias, Biomateriales y Nanomedicina: 2006-2008
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Fernando Albericio's group, March 2006.

Nucleic acids chemistry

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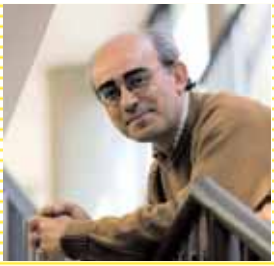

Ramón Eritja

Nucleic acids (DNA and RNA) formed by long chains of nucleotides play an important role in the genetic inheritance. In the lab, small versions of nucleic acids known as oligonucleotides, can be prepared. Our research focuses on the methodology used for the preparation of oligonucleotides and related compounds as well as the study of their properties.

Synthesis of oligonucleotide conjugates

Oligonucleotides are essential tools for DNA detection and manipulation. At the end of the eighties, the discovery of the polymerase chain reaction (PCR) and the use of oligonucleotides as inhibitors of gene expression triggered a high demand for oligonucleotide derivatives with new, tailored properties, in addition to their hybridisation properties. Most of these new properties were achieved by the addition of special molecules to oligonucleotides, which results in oligonucleotide conjugates.

Synthesis of oligonucleotide-peptide conjugates

The preparation of oligonucleotide-peptide conjugates poses an interesting challenge since conventional protection schemes are not compatible. In this regard, all standard protection schemes in solid-phase peptide synthesis use acid treatments which could cause partial depurination of DNA. Two strategies that overcome these problems have been described: 1) the post-synthetic conjugation approach; and 2) the stepwise solid-phase synthesis approach.

In the stepwise approach, these conjugates are prepared by stepwise addition of amino acids and nucleobases in solid phase on the same solid support. This is usually performed by first assembling the peptide by means of *t*-butoxycarbonyl (Boc) -protected amino acids with base labile groups, such as fluoren-9-ylmethyl (Fm), fluoren-9-ylmethoxycarbonyl (Fmoc), and trifluoroacetyl (TFA) groups, to protect side chains. Although these protocols generate oligonucleotide-3'-peptide, they cannot be used to incorporate peptides at the 5' end or in the middle of the oligonucleotide sequence. We have developed an efficient method to synthesise short oligonucleotide-5'-peptide conjugates via stepwise synthesis using commercially available Fmoc-protected amino acids (Ocampo *et al*, 2005). Although the Fmoc-removal conditions are not orthogonal to the base-labile cyanoethyl protection of DNA phosphotriester moieties, we found that the presence of unprotected phosphate groups did not hinder the assembly of small peptide sequences on oligonucleotide supports. Groups labile to mild acidic conditions (such as 1% trifluoroacetic acid) were used to protect the side chains of Nα-Fmoc-protected amino acid (Ocampo *et al*, 2005).

Synthesis of oligonucleotides conjugates carrying lipids, steroids and carbohydates

Modified oligonucleotides are being used to inhibit gene expression. In order to enhance the activity of oligonucleotides and their analogues, they have been covalently linked to intercalating, alkylating, photocrosslinking and radical-generating reagents. In addition to increasing affinity for the target sequence, some of these compounds promote the uptake of oligonucleotides by cells and improve their resistance to nucleases. Lipid moieties, such as cholesterol to oligonucleotides, enhance the antisense activity of these compounds as well as the silencing properties of small interfering RNA (siRNA). This year we have prepared several oligonucleotide (DNA and RNA) conjugates carrying lipids, steroids and carbohydrates. The properties of these new compounds are currently being studied. We are collaborating with several groups in this field. The group led by Jose Carlos Perales is working on the evaluation of the inhibitory properties of conjugates *in vivo*, while that of Juan Carlos Morales is preparing the carbohydrate derivatives needed for solid-phase synthesis. We are also involved in a collaborative project with Sylentis-PharmaMar. Oligonucleotide-steroid conjugates are also being used for the development of bioanalytic

devices for anti-doping and food control of illegal steroidal anabolic hormones, in collaboration with Pilar Marco and Josep Samitier.

Synthesis of oligonucleotide clamps for triplex formation

Oligonucleotides interact in a sequence-specific manner to homopurine-homopyrimidine sequences of duplex and single-stranded DNA and RNA to form triplexes. Nucleic acid triplexes have potential applications in diagnostics, gene analysis, and therapy. Depending on the composition and orientation of the third strand vis-à-vis the central homopurine Watson-Crick strand, triplexes are classified into two main categories: (i) parallel and (ii) antiparallel. The most well characterised parallel triplex is that formed between a double-stranded homopurine-homopyrimidine helix (duplex DNA) and a single-stranded homopyrimidine track (triplex-forming oligonucleotide). In this type of triple helix, the triplex-forming oligonucleotide binds to the major groove (parallel to the homopurine strand of Watson-Crick double-helical DNA) via Hoogsteen hydrogen bonding, and is stabilised under acidic conditions. In the antiparallel triplexes, the third strand, composed of purine bases binds in a pH independent and antiparallel fashion to the homopurine duplex strand via reverse-Hoogsteen hydrogen bonds

Our group, in collaboration with Modesto Orozco, has found that the introduction of an amino group at position 8 of the Watson-Crick purine produces a high stabilisation of parallel triplexes. The triplex-stabilisation properties of the amino group at this position results from a combined effect of the gain of one Hoogsteen purine-pyrimidine H-bond and the propensity of the amino group to be integrated into the 'spine of hydration' located in the minor-major groove of the triplex.

Sequence-specific triple-helix structures can also be formed by DNA clamps. Parallel-stranded DNA clamps consist of purine residues linked to a homopyrimidine chain of inverted polarity by 3'-3' or 5'-5' internucleotide junctions, which interact with single-stranded homopyrimidine nucleic acid targets. In this triplex, the homopurine strand of the clamp binds the homopyrimidine target through Watson-Crick bonds; and the homopyrimidine strand of the clamp forms the triplex via Hoogsteen bonding. We have found that the stability of triple helices is enhanced by replacing natural bases with several modified bases, such as 8-aminopurine residues.

This same strategy has been proven to generate stable antiparallel triplexes using purine-rich clamps as templates (*ie*, antiparallel-clamps). Antiparallel clamps formed by a G,A- or G,T-Hoogsteen sequences linked to a polypurine sequence carrying 8-aminopurines have also been prepared and shown to form more stable triplexes if 8-aminoguanine is present in the purine Watson-Crick position.

The increased binding properties of clamps carrying 8-aminopurines may be of particular interest for the development of applications based on triple-helix formations. Specifically, we have developed capture probes for the detection of bacteria in food. Figure 1 shows an outline of the capture assay. In a previous study, we observed that binding of oligonucleotide clamps was prevented by the target's secondary structure. This problem was solved with a new strategy based on the addition of a tail sequence to a Watson-Crick strand, such that modified clamps formed triple helices with structured DNA or RNA molecules. This tail-clamp strategy overcame structural interferences, while simultaneously greatly increasing the stability of triplex formation. We synthesised parallel tail-clamps designed to bind to *Listeria innocua iap* mRNA sequences containing a polypyrimidine track (Nadal *et al*, 2006). Our aim was to obtain optimum conditions for the triplex affinity capture of these sequences in order to develop new detection methods for pathogens, by means of the specific identification of their nucleic acids. In our study, we explored the effects of pH on the interaction of parallel tail-clamps with their target by UV thermal melting analysis. In addition, we optimised a triplex affinity capture assay capable of recovering *iap* mRNA molecules from a total RNA solution purified from *L. innocua* cells in a sequence-specific manner. Optimal results were obtained with tail-clamps carrying 8-aminoadenine moieties under neutral pH conditions: 45% of the *iap* mRNA molecules from a total RNA solution were captured (Nadal *et al*, 2006).

Oligonucleotides and nanotechnology

There is a considerable interest in the use of biopolymers (peptides, proteins and nucleic acids) for the assembly of nanomaterials. Moreover oligonucleotides linked to nanoparticles are being used to monitor DNA hybridisation as well as to detect a nucleic acid sequence of interest. We are collaborating actively with several groups in this area. The following results have been obtained.

A method for the fabrication of gold nanostructures using oligonucleotide derivatives

From the fabrication of the first chips in the 1950s to the present the density of the functional units per surface unit has doubled every 3-4 years. This has been possible thanks to technological advances that allow the miniaturisation of circuits. However, the current technologies derived from photolithography

are reaching their physical limits while, conversely, chemists and biologists are working with larger and larger biomolecules. For these reasons, it is believed that in the near future, circuitry may be prepared using biomolecules that will order the nanomaterials in solution. The resulting structures will then be settled into the spaces produced by photolithography.

One promising biomolecule is DNA since it forms lineal structures composed of two strands with known self-assembling properties. Moreover, there is a solid methodology to prepare DNA strands of up to 100 bases (about 30 nm of length).

One line of research of particular interest is the preparation of synthetic DNA derivatives designed to assemble a molecular wire between gold electrodes, which are required to address individual nanoparticles from macroscopic electrodes. We have used synthetic oligonucleotides to prepare the molecular wires, thereby offering the possibility to introduce modifications at any predetermined position.

The proposed structure is shown in Figure 2 and consists of three elements with distinct roles: anchoring, extension, and recognition. Two anchoring elements are located at each end, both with disulfide groups

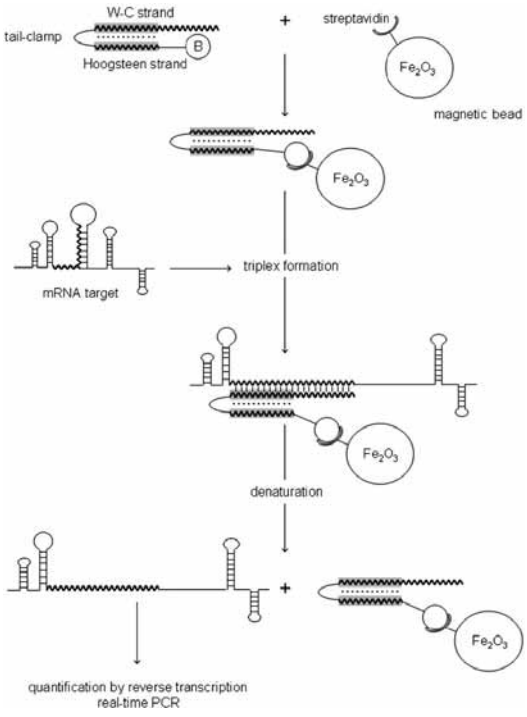

***Figure 1**. Triplex-mediated capture of Listeria RNA (Nadal* et al*, 2006)*

that allow the wires to be attached to the electrodes. The centre of the structure is a chimeric compound with a DNA segment that positions the element in the middle of the structure. It also contains biotin as a recognition group, isolated from the DNA by a spacer molecule comprising two hexaethylenglycol units. This recognition element is used to direct a nanoparticle into the middle of the structure as well as to connect the two branches. The size of the whole structure is determined by the extension elements between the recognition and the anchoring elements.

The extension elements (100 bases long) were prepared using protocols to produce long oligonucleotides. Special protocols were developed for the preparation of the recognition elements since the polarity of the DNA strands is reversed in the middle of the molecule, thereby providing symmetry to the central assembly. The two-armed recognition element was prepared by sequentially adding 10 different phosphoramidites. Starting from the 3'-end, the first half of the sequence was assembled using the four standard phosphoramidites. Subsequently, hexaethyleneglycol and biotin-tetraethyleneglycol phosphoramidites were added. Finally, the second half of the molecule was assembled using the four reversed phosphoramidites. Synthesis of the oligonucleotides carrying three branches was conducted in similar fashion although a symmetric branching molecule was added. First, the 20 mer sequence was built in the 3'->5' direction using standard phosphoramidites and the hexaethyleneglycol phosphoramidite. Biotin-tetraethyleneglycol was thenadded. Afterwards, a symmetric branching phosphoramidite was added to the sequence. Finally, the rest of the desired sequence was assembled in the 5'->3' direction using reversed phosphoramidites and the hexaethyleneglycol phosphoramidite.

Using these oligonucleotides the DNA-templated assembly of a protein-functionalised 10 nm gap electrode, from suitably modified gold nanoparticles on a silicon wafer substrate, was achieved (Stanca *et al*, 2006). This protein-functionalised electrode was recognised and selectively bound by a suitably modified gold nanoparticle that was localised in the 10 nm gap (Stanca *et al*, 2006). These findings are of interest for the fabrication of next-generation electronic devices.

Immunodetection of oligonucleotide-peptide conjugates by induced-coupled plasma mass spectroscopy (ICPMS)

ICPMS has been used to study the atomisation of nanoparticles for the development of a novel non-isotopic immunoassay by coupling this technique with the sandwich-type immunoreaction. ICPMS is an out-

standing method for trace element determination and is gaining wide acceptance due to its extremely high sensitivity and element specificity. In collaboration with Arben Merkoçi, we have explored for first time the use of ICPMS for DNA monitoring through the detection of metallic nanoparticles. The enhanced DNA signals obtained by ICPMS of gold tags are combined with the high specificity of oligonucleotide-peptide conjugate interactions with anti-c-myc monoclonal antibody followed by immunoreaction with the secondary antibody (Anti-mouse IgG) conjugated to gold nanoparticles. Although in a very early phase, the proposed ICPMS-linked DNA assay may have significant potential as a non-isotopic DNA detection method for the simultaneous determination of various sequences by labelling several kinds of inorganic nanoparticles and also by taking advantage of the recent development of ICPMS technique. This study will also be of interest for the development of novel genosensors and DNA chips based on multiple labelling by the specific immunoreactions with the peptide sequences introduced into the DNA probes (Merkoçi *et al*, 2005).

Development of new drugs that bind DNA

Drug development has traditionally focused on active sites of proteins, and on identifying molecules, such as inhibitors, that bind to these active sites and directly block directly interactions with natural substrates. In addition to this direct mode of enzymatic regulation, nature makes extensive use of drug interaction with nucleic acids that have become important antibiotic, antiviral and anticancer agents. In collaboration with Fernando Albericio (IRB Barcelona) and Crystax Pharmaceuticals, we are designing and synthesising new drugs that interact with DNA. In this project, we are applying the knowledge gained from peptide and oligonucleotide synthesis to obtain new and larger molecules in order to increase affinity for a particular DNA site.

Oligonucleotides of biological and structural interest

Synthesis of oligonucleotides carrying DNA methyltransferase inhibitors

Aberrant DNA methylation is a common finding in cancer. Several drugs that inhibit DNA methylation are active against some malignancies. The cytosine analogues, 5-azacytidine and 5-aza-2'-deoxycytidine, are the most frequently studied inhibitors of DNA methylation. Zebularine (1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one), another pyrimidine analogue which lacks the 4-amino group of the other cytosine analogues, has been shown to inhibit DNA methylation and may have activity against cancer. Zebularine has the advantage of being very stable, and can be administered orally. However, zebularine has the disadvantage of being a less potent inhibitor of DNA methylation compared to azacytosine derivatives. It is believed that zebularine is not metabolised to its triphosphate form as efficiently as 5-azacytidine and 5-aza-2'-deoxyctyidine, and therefore it is not efficiently incorporated into DNA. In collaboration with Victor Márquez and Allen Yang, we have prepared oligonucleotides carrying 2'-deoxyzebularine in order to measure the efficiency of incorporation in response to the drug. When zebularine was included in the template strand, dGTP was preferentially incorporated by the Klenow fragment opposite zebularine, but dATP and dTTP were incorporated with 8.1% and 5.2% the efficiency of dGTP. In addition, zebularine in the template strand was noted to inhibit DNA extension. Thus dZTP is efficiently incorporated into DNA and acts as a cytosine analogue, which is consistent with its capacity to inhibit DNA methylation.

Oligonucleotides and G-quadruplex

G-rich oligonucleotides may form intra- or intermolecular structures involving the formation of G tetrads. These structures are naturally present at the end of chromosomes or telomeres. Recently, G-quadruplex structures have been found in promoter regions of oncogenes and several authors have proposed that the G-quadruplex regulates the expression of these proteins. Due to the potential biological relevance of the G-quadruplex, we have initiated the study of

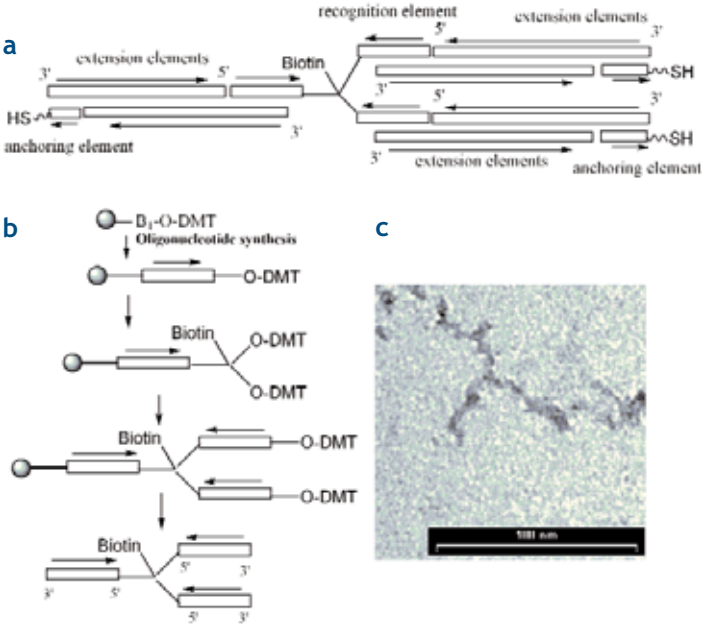

Figure 2. *a) Targeted 3-branch oligonucleotide assembly used as template for gold nanostructure containing a biotin molecule used to direct a streptavidine molecule to the middle of the structure (Stanca* et al*, 2006); b) outline of the synthesis for the three-branched recognition elements; c) Transmission electron microscopy (TEM) image of three-branched DNA negative stained with uranyl acetate 1%.*

these structures using synthetic oligonucleotides. In collaboration with Raimundo Gargallo, we have studied a bimolecular quadruplex. Specifically, the resolution of the dimeric intermolecular G-quadruplex/duplex competition of the telomeric DNA sequence 5'-TAG-GGT-TAG-GGT-3' and its complementary 5'-ACC-CTA-ACC-CTA-3' was examined. Melting experiments of both sequences and their mixtures were monitored by molecular absorption, molecular fluorescence and circular dichroism spectroscopy. Molecular fluorescence measurements were carried out using the molecular beacons technology, in which the 5'-TAG-GGT-TAG-GGT-3' sequence was labelled with a fluorophore and a quencher at the ends of the strand. It was observed that the parallel G-quadruplex is more stable than the antiparallel G-quadruplex. When the complementary C-rich strand is present, a mixture of both G-quadruplex structures and Watson-Crick duplex is observed, the duplex being the major species (Jaumot *et al*, 2006).

The formation of G tetrads is not desired when designing triplex-forming oligonucleotides (see above), because the formation of G-tetrads may reduce the formation of triplex. It has been described that the quadruplex inhibits triplex formation, especially the antiparallel triplex, because of the presence of a large number of guanines in the oligonucleotides. This has fuelled the design of modified nucleobases that destabilise the tetraplex. However, unfortunately in most cases, these nucleobases also destabilise the triplex. Given the large triplex-stabilising effect of 8-aminoguanine, we tested whether G tetrads were affected by the presence of 8-aminoguanine residues. As a model of quadruplex-forming oligonucleotides, we selected the 15-base long thrombin aptamer 5'-GGTTGGTGTGGTTGG-3'. It was shown that this oligonucleotide adopts a monomeric chair quadruplex structure in the presence of potassium, which is characterised by a denaturation-renaturation profile that is reversible and observable by several techniques. Using this model oligonucleotide we have demonstrated that 8-aminoguanine has a unique profile as a molecule that simultaneously shows strong triplex-stabilising and quadruplex-destabilising properties (Lopez de la Osa *et al*, 2006).

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RESEARCH NETWORKS AND GRANTS

Precision chemical nanoengeneering: integrating top-down and bottom-up methodologies for the fabrication of 3-D adaptive nanostructures architectures (Nano-3D)
European Commission, STREP, NMP4-CT2005-014006: 2004-2007
Project Coordinator: Jon Preece

Development of nanobio-analytical platforms base don molécula recognition using optical and/or electrical detection, (Nanobiomol)
Ministry of Education, Strategic action on nanotecnology, NAN2004-09415-C05-03: 2005-2008
Project Coordinator: Josep Samitier

Research groups of Catalonia. Group of synthesis and structure of biomolecules
Comission of University and Research, Autonomous Govern of Catalonia (2005SGR00693): 2006-2008
Project Coordinator: Enrique Pedroso

Design and functionality of non-linear electrochemical nanoscale devices (Dynamo)
European Commission, STREP, NEST-2004-ADV proposal 028669-1: 2006-2008
Project Coordinator: Kyösti Kuntturi

Development of new nanosensors functionalised with DNA
AECI-MEC, Tunez-Spain collaborative project (A/2673/05): 2006
Project Coordinators: ZM Bacca and A Errachid

In addition the group is active in the following networks:
- RIBORED, a network of Spanish scientist working in RNA
- RANN, a network of Spanish scientists working in nucleosides, nucleotides and nucleic acids
- NANOSPAIN, a network of spanish scientists working in nanosciences
- PLATAFORMA ESPAÑOLA DE NANOMEDICINA, a network of Spanish scientists working in nanomedicine
- EUROPEAN PLATFORM ON NANOMEDICINE, a network of European scientists working in nanomedicine

OTHER FUNDING SOURCES

Synthesis and properties of modified oligonucleotides with potential anticancer activity
Fundación La Caixa (BM04-52-0): 2004-2007
Project Coordinator: Ramón Eritja

Modified oligonucleotides for the study of triplex formation and for obtaining other structures with potential technological and structural interest
Ministry of Education, BFU-2004-02048/BMC: 2004-2007
Project Coordinator: Ramón Eritja

COLLABORATIONS

Synthesis of oligonucleotides with structural interest
Raimundo Gargallo (University of Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps
Carlos González (Institute of Structure of Matter-CSIC, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps
Anna Nadal (University of Girona, Spain)

Research on nanosensors
Arben Merkoçi (National Centre on Nanotechnology, Barcelona, Spain)

Synthesis of oligonucleotides active against AIDS
Maria Angeles Muñoz Fernández (Hospital Gregorio Marañón, Madrid, Spain)

Synthesis of oligonucleotides
José Carlos Perales (University of Barcelona, Spain)

Synthesis of oligonucleotide-carbohydrate conjugates
Juan Carlos Morales (Institute of Chemical Research, Sevilla, Spain)

Synthesis of oligonucleotide-peptide conjugates
José Luis Mascareñas (University of Santiago de Compostela, *Spain)*

Synthesis of oligonucleotide-esteroid conjugates
Pilar Marco (IIQAB-CSIC, Spain)

Research on nanosensors functionalised with oligonucleotides
Josep Samitier (IBEC, Parc Científic de Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps
Carlos Cuidad (University of Barcelona, Spain)

Synthesis of oligonucleotides carrying methyltransferase inhibitors and conformationally-restricted nucleosides
Victor Marquez (National Institutes of Health, Bethesda, USA?)

Synthesis of oligonucleotides for the assembly of nanomaterials
Donald Fitzmaurice (University College Dublin, Ireland)

Synthesis of oligonucleotide-peptide conjugates, synthesis of new drugs that binds DNA, synthesis of new RNA derivatives
Fernando Albericio (IRB Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps
Modesto Orozco (IRB Barcelona, Spain)

Synthesis of new drugs that bind DNA
Crystax Pharmaceuticals (Barcelona, Spain)

Synthesis of new RNA derivatives
Sylentis-PharmaMar (Madrid, Spain)

Ramón Eritja's group, March 2006.

Design, synthesis and structure of peptides and proteins

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Ernest Giralt

"Give us the three-dimensional structure of a given protein, for example, an interesting therapeutic target; put your finger in a given surface patch; and we will be able to design for you a very efficient, selective and protease resistant (peptide or non-peptide) ligand." This sentence is still more a dream than a reality, but it reflects one of the most important research lines developed in our laboratory. Improving our knowledge of the rules that govern molecular recognition is clearly, behind all our endeavours in this field. With this aim, we study protein-protein interactions in general and protein self-assembly in particular. Our lines of research not only contribute to enhancing our knowledge of molecular recognition mechanisms, but also provide numerous opportunities in terms of drug discovery. However, many additional unknowns remain to be addressed in order to advance in this field. Several of these are also the focus of our research activities: how can we design a peptide to ensure efficient cellular uptake?; it is possible to remotely control the disruption of amyloid fibrils?; can we use peptides to shuttle drugs across the blood-brain barrier? Finally, methodological improvements are constantly required for all scientific activities. This is the focus of our more recent work in NMR for conformational analysis, improving solid-phase peptide synthesis or developing computational evolutionary algorithms for drug discovery.

Protein-protein interactions

The challenge of dividing finite geometric objects into isometric segments has fascinated structural and synthetic chemists for decades. In a seminal paper published in 1983, Mislow and colleagues analysed the intriguing way of cutting an apple known as "*la coupe du roi*". Figure 1 illustrates how a pair of homochiral segments can be obtained from an achiral object (in this case, an apple) by first making two vertical half-cuts, one from the top to the equator and the other from the bottom to the equator, followed by two non-adjacent horizontal cuts. Although this concept has been applied to the retrosynthetic analysis of highly symmetric organic compounds, such as fullerene C(60) or cyclic diketones, as well as to helical organometallic compounds, to the best of our knowledge it has never been applied to proteins.

Rabbit uteroglobin (UG) can be dissected into two identical homochiral halves either by the conventional reduction of the two disulfide bridges or via "*la coupe du roi*". In the former case, which has been extensively studied in the literature and probably occurs in determined physiological conditions, two identical HS- (1,2,3,4)-SH dithiol 70mers are formed. In the latter, reported for the first time in our publication (Nicolás *et al*, 2005), two identical homochiral halves are also formed (*ie*, α(1,2)-S-S-α(3,4) disulfide 70mers). Independently of how UG is "dissected", the two identical UG halves form a globular noncovalent dimer, the folding of which is most likely driven by interhelical interactions. The importance and specificity of these interactions are highlighted by

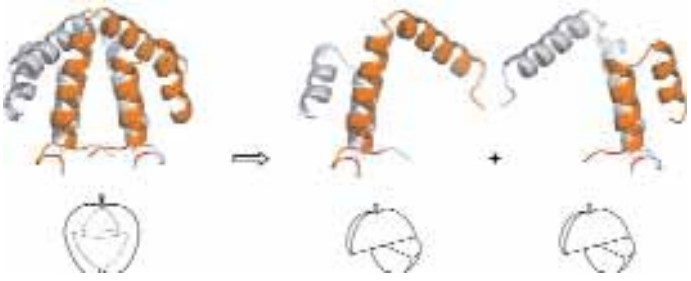

Figure 1. "Coupe du roi" *bisection of proteins. Spontaneous tetramerisation of two peptides that span the sequence of the rabbit uteroglobin monomer.*

the ability of a mixture of α(1,2)-SH and α(3,4)-SH to regioselectively form α(1,2)-S-S-α(3,4) and, therefore, to form the noncovalent 140mer by spontaneous oxidation in aqueous solution. The results obtained in this study suggest that the "*coupe du roi*"-nicked UG has interesting molecular recognition properties, suggesting its potential as a utile, readily synthesised framework for the design of biologically active molecules.

This study with UG is but only one example of the five systems that we are currently working on a two-fold interest: to improve our understanding of protein-protein recognition and to modulate the stability of protein-protein aggregates.

Like UG, HIV-protease (HIV-1 Pr) is also a dimer, but in this case non covalent. We have recently developed an efficient NMR method for the characterisation in solution and at atomic level of the interaction of enzyme inhibitors with a mode of action based on inhibiting dimerisation (Frutos *et al*, 2007). Also with HIV-1 Pr, we have developed an extremely robust ^{19}F-NMR-based method for screening protease inhibitors (Frutos *et al*, 2006).

The B-domain of *S. aureus* Protein A is our benchmark for the design of helical peptides based on D-amino acids with the capacity to retain the same side-chain topology as helices made using native L-amino acids. D-amino acids are also the focal point of our efforts to design efficient amyloid aggregation inhibitors. Last year, in collaboration with Chris Dobson and Carol Robinson in Cambridge (Carulla *et al*, 2005), we reported in *Nature* how the individual protein copies from an amyloid fibril recycle efficiently from the fibril to the solution and back to the fibril. These findings are very promising for the design of synthetic amyloid aggregation inhibitors. Our efforts in this direction are well advanced.

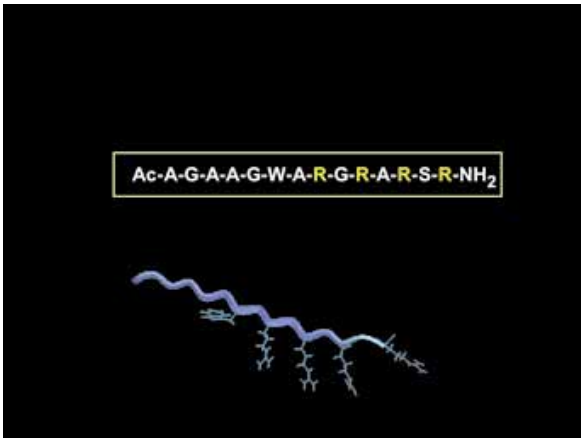

***Figure 2**. Chemical structure of CAN4, a synthetic ligand that interacts with the P53 tetramerisation domain.*

Protein-surface recognition

Targeting protein surfaces that are involved in protein-protein contacts is among the most promising strategies for the development of new drugs. These contacts are key in almost all biological processes, and ligands that modulate such contacts would be highly valuable tools for the treatment of diseases. Nevertheless, protein surfaces have only recently been exploited as therapeutic targets, and many research groups are now searching for compounds that specifically recognise given areas of protein surfaces. These molecules could then be used for therapeutic applications, such as tools for chemical genetics studies, or to impart chaperone-like effects to stabilise the native conformation of a protein and/or rescue destabilised mutants. (See Figure 2.)

Surfaces involved in protein-protein interactions are typically large and flat, and, compared to cavities such as enzyme active sites, are highly solvated and rich in polar residues. Small molecules require surface pockets in order to bind to proteins with high affinity, and this hampers their capacity to disrupt protein-protein interactions. Although promising candidates have recently been obtained by screening libraries of small compounds, the principles for integrating surface recognition into the preliminary design of compounds have yet to be established.

NMR studies have been previously used by our group to demonstrate that a nonpeptidic tetraguanidinium compound recognised an anionic patch on the surface of the tetramerisation domain of p53 in aqueous solution with a K_D of 50 μM (Salvatella *et al*, 2004). We have now reported (Martinell *et al*, 2006) a designed peptide ligand (Figure 2) that interacts with the same anionic patch on the surface of the p53 tetramerisation domain in aqueous solution. Using combinatorial chemistry, we have examined the different moieties that participate in the mutual recognition of the designed peptide CAN4 and the p53 tetramerisation domain. The conclusions from this study could be useful for the design of ligands directed to other highly anionic protein-surface patches.

P53 is, again, only one of the systems that we are currently examining from the point of view of the design of binding ligands. Among these other proteins, we are devoting considerable effort to proline oligopeptidase (POP). POP is a non-validated therapeutic target for central nervous system (CNS) disorders, including schizophrenia and bipolar disorder. We have recently reported the use of a new ^{19}F-NMR-based screening method for POP inhibitors present in

aqueous plant extracts, which we have prepared starting from about 50 plants used in traditional Chinese medicine for the treatment of CNS-related disorders (Tarragó *et al*, 2006; Figure 3). An alkaloid, berberine, was identified as a new POP inhibitor and we are now working on the synthesis of berberine derivatives in order to improve the activity and selectivity of this compound. The screening of Chinese medicinal plants is one of the approaches that we are pursuing in our search for new POP inhibitors. Others include structure-based drug design and combinatorial chemistry. (See Figure 3.)

Cell-penetrating peptides

In recent years, cell-penetrating peptides (CPPs) have proven to be an efficient intracellular delivery system. The mechanism for CPP internalisation, which first involves interaction with the extracellular matrix, is followed in most cases by endocytosis and finally, depending on the type of endocytosis, an intracellular fate is reached (Pujals *et al*, 2006).

CPPs are considered potential vectors to carry drugs that have low bioavailability across cell membranes. Properties, such as amphipathicity, high guanidinium content, and hydrophobicity, have been reported to be essential for a peptide to be able to cross a cell membrane. A new family of CPPs of the general formula $(VRLPPP)_n$, in which two of these features, amphipathicity and arginine groups, have been imprinted on a polyproline sequence, was reported by our group some time ago (Angew Chem Int Ed, 2004). The key to the design of these compounds was to maintain a proline content of at least 50% to ensure that the molecules adopt a left-handed polyproline II (PP II) helical structure in solution with a periodicity of 3.0 residues per turn. The remaining 50% of residues were optimised for amphipathicity of the PP II helix. More precisely, hydrophobic Val and Leu residues were placed at 1, 3, 7, 9, ... positions, while polar Arg residues were placed at 2, 8, ... positions. Ultimately, a new family of non-cytotoxic peptides with good cellular uptake properties was obtained (Sweet Arrow Peptides). We are now studying whether the amphipathicity of the PP II helix of these compounds could be further increased by manipulation of the Pro-containing regions. Specifically, it was thought that replacement of a Pro residue located at the hydrophobic face of a PP II helix with another amino acid might increase the amphipathicity and/or enhance the cellular uptake properties of a given CPP. The synthesis of γ-(dimethylsila)proline, or silaproline (Sip), as a proline derivative with enhanced hydrophobicity, was recently reported by Cavelier *et al*. (J Am Chem Soc, 2004). We considered that this non-natural amino acid could be used for such a purpose. The most efficiently

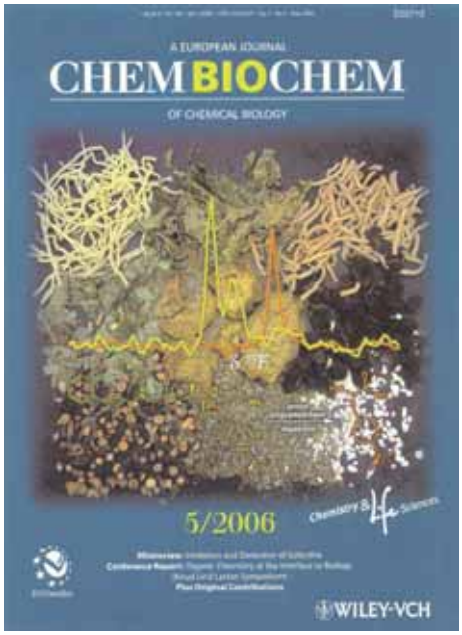

***Figure 3**. Cover of the ChemBioChem issue containing the article 'Identification of traditional Chinese medicinal plants with oligopeptidase activity using ^{19}F-NMR' (Frutos* et al*, 2006). The fluorine spectra of the fluorinated substrate (in yellow) and the fluorinated product (in orange) are shown over a background of some of the plants used in our study.*

internalised Pro-rich CPP [CF-$(VRLPPP)_3$, (1)] was chosen as a reference. Peptide 2 [CF-VRLPPSip$(VRLPPP)_2$ p_2] was designed by substituting Sip for Pro, which is located on the hydrophobic face of the amphipathic PP II helix. Using flow cytommetry, we have shown that, in Hela cells, replacement of a proline with silaproline causes a 20-fold increase in the cellular uptake of the proline-rich peptide. In addition, we have demonstrated that replacement of a Pro by Sip on the hydrophobic face of a Pro-rich amphipathic peptide does not perturb secondary structure or prevent peptide aggregation, and greatly enhances the cellular uptake of the peptide. In addition to highlighting the relevance of amphipathicity in CPP design, these results also emphasise the utility of Sip as a new source of amphipathicity (Pujals *et al*, 2006).

Peptide-nanoparticles: an opportunity for the remote control of protein self-assembly

Misfolded proteins self-assemble into insoluble fibrous deposits, thereby causing diseases called amyloidosis (Alzheimer's, Parkinson's, Huntington's, and type II diabetes among others). During self-assembly, there is an equilibrium between soluble monomer and increasingly larger insoluble aggregates, fibrils, which entangle and precipitate, forcing

the equilibrium toward aggregated forms. Formation of these fibrils requires energy (for example, in the form of stirring), but once formed, they are stable. Vigorous shaking and sonication, high hydrostatic pressure, and temperature cycling reverse the natural equilibrium and redissolve the precipitates. In collaboration with Marcelo Kogan and Victor Puntes, from the Universidad de Chile and Institut Català de Nanotecnologia respectively, we have addressed the combined use of gold nanoparticles and microwave irradiation for the remote manipulation of this aggregation process (Kogan *et al*, 2006).

The local heat delivered by metallic nanoparticles selectively attached to their target can be used as molecular surgery to safely remove toxic and clogging aggregates. We have applied this principle to protein aggregates, in particular to the beta-amyloid protein involved in Alzheimer's disease, a neurodegenerative disease where unnaturally folded proteins self-assemble and deposit forming amyloid fibrils and plaques. We have shown that it is possible to remotely redissolve these deposits and to interfere with their growth, using the local heat dissipated by gold nanoparticles selectively attached to the aggregates and irradiated with low gigahertz electromagnetic fields. We have used peptide sequences covalently attached to the surface of the nanoparticle as an anchor to ensure the attachment of the nanoparticle to the target protein aggregate. Simultaneous tagging and manipulation by functionalised gold nanoparticles of beta-amyloid protein at several stages of aggregation allow non-invasive exploration and dissolution of molecular aggregates.

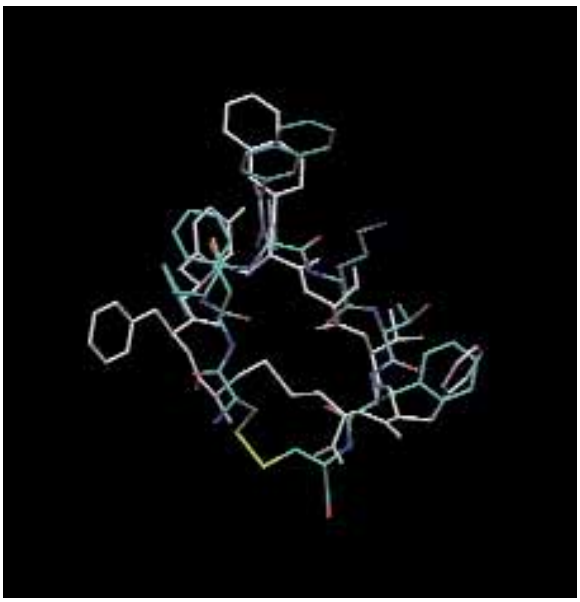

***Figure 4**. NMR 3D structures of the major and minor conformational isomers of a potent SSTR3-selective analogue of somatostatin.*

Conformational analysis

Conformational analysis of peptides and proteins is a key issue when addressing relationships between structure and biological activity. In collaboration with Fernando Albericio's group, our activity in this field focuses on NMR studies of cyclic peptides and depsipeptides, very often of marine origin, which show promising activities as anti-tumour agents (Cruz *et al*, 2005a,b)

In addition, in collaboration with Jean Rivier's laboratory at The Salk Institute in San Diego (California), we have also undertaken the conformational analysis of a group of new cyclic peptides which show activities of interest in the CNS.

Peptides play a major role in signalling processes in the CNS. The same molecule frequently interacts with many different receptors carrying out several biological functions. One of the most paradigmatic cases illustrating this behaviour is that of somatostatin (SRIF), H-Ala^{1}-Gly^{2}-c[Cys^{3}-Lys^{4}-Asn^{5}-Phe^{6}-Phe^{7}-Trp^{8}-Lys^{9}-Thr^{10}-Phe^{11}-Thr^{12}-Ser^{13}-Cys^{14}]-OH, a cyclic tetradecapeptide that acts as a neurotransmitter and neuromodulator in the CNS, as an inhibitor of the release of numerous hormones and as a regulator of cell proliferation and differentiation. SRIF establishes high-affinity interactions with a family of at least five receptor subtypes, SSTR1-5. Although all receptors share common signalling pathways, they have specific functional roles and some biological responses show subtype selectivity. For example, SSTR2 mediates mainly the inhibition of the release of glucagon and growth hormone. SSTR5 controls insulin secretion, and both SSTR2 and SSTR5 mediate the antiproliferative effects of somatostatin on cellular growth processes in tumours. However, the individual functions of the somatostatin receptors *in vivo* are still not fully understood.

Given its wide range of physiological functions, SRIF is a target for the development of receptor subtype-specific analogues. Hundreds of somatostatin analogues that bind with some selectivity to receptor subtypes are currently available and extensive structural studies have been done to ascertain the minimum structural requirements of the analogues for selective binding. To date, a distinct pharmacophore model has been proposed for analogues binding predominantly to SSTR1, SSTR2/SSTR5 and SSTR4 receptors. It appears that the consensus structural motif for these selective ligands requires a unique arrangement of several side chains, which are critical for selective binding. For SSTR3-selective analogues, no

structural information is available to elucidate their binding affinity and selectivity.

A study on cyclic peptides using high field NMR (800MHz) in water solution and molecular dynamics calculations describes, for the first time, the 3D structure of a potent SSTR3-selective analogue of somatostatin. From these data a structural motif has been proposed that could be responsible for SSTR3 selectivity (Gairí *et al*, 2005; see Figure 4.)

New methods in peptide and protein synthesis

Polymers made of amino acids (polyamino acids, peptide copolymers) are emerging as promising therapeutic compounds. These polymers have widespread applications in the field of drug delivery. Drugs are physically entrapped within the polymer matrix or chemically conjugated to the polypeptide for slow release in the biological milieu. A milestone example of therapeutic polyamino acid carriers is paclitaxel polyglumex, a biologically enhanced version of taxol conjugated to a polyglutamate polymer that has recently been *fast track* designated by the FDA for the treatment of advanced non-small cell lung cancer in women. In this context, polyproline polymers are also attracting much attention because of their therapeutic potential. Polyproline is soluble in water, and thus, has been used to solubilise poorly water-soluble proteins obtained by recombinant techniques. Most of these proteins, such as interferons and interleukins, are of high therapeutic interest. Polyproline polymers have also found use in affinity chromatography for the purification of platelet profilin. Recently, dendrimers composed of polyproline branches have been shown to be actively internalised by rat kidney cells and to entrap the antibiotic ciprofloxacin.

Polyamino acids are most conveniently synthesised by polymerisation of the corresponding amino acid *N*-carboxyanhydride. However, the case of proline is unique among coded amino acids as the α-amino group is bound to the side chain, thereby yielding a cyclic secondary amine (pirrolidine) and showing some conformational restrictions. These features probably underlie the poor synthetic yields obtained using currently available methods for α-amino acid N-carboxyanhydride (NCA) formation. Generally speaking, NCAs are achieved by treatment of the corresponding amino acid with phosgene, the so-called Fuchs method. In the case of proline, in contrast to other amino acids, the *N*-carbamoyl intermediate does not cyclise spontaneously, and the use of a non-nucleophilic base, typically a tertiary amine, is required for cyclisation to the NCA.

Procedures described in the literature require slow addition of solutions of phosgene at low temperature and the use of tertiary amines, such as triethylamine, which are difficult to remove and appear in variable amounts in the final crystallised proline NCA product. We are working on the preparation of Pro-NCA in high yields and purities using solid triphosgene and polymer-bound bases. We have recently reported a new procedure for the preparation of proline NCA in high yield and purity using polymer-supported tertiary amines. The polymer-supported amine can be recycled with a basic wash and filtration of the resin. The procedure facilitates the efficient preparation of polyproline polymers of potential therapeutic interest (Gulin *et al*, 2006; see Figures 5 and 6.)

Computer-aided molecular design

One of the goals of computational chemists is to automate the *de novo* design of bioactive molecules. Despite significant advances in computational approaches to ligand design and binding energy evaluation, novel procedures for ligand design are required.

Several research groups currently focus on the development of methodologies for the design of peptidic drugs. Structure-based drug design is an example of an effective technique, whereby the design process is tackled as an engineering problem, and high-throughput screening (HTS) of numerous compounds from combinatorial libraries is performed against a known target. We have recently proposed (Belda *et al*, 2005) a new *in silico* approach, dubbed ENPDA (Evolutionary structure-based *de Novo* Peptide Design Algorithm), which is a hybrid of the two aforementioned strategies. This approach allows the screening of large numbers of candidate peptides that are derived from a semi-rational process involving evolutionary computation.

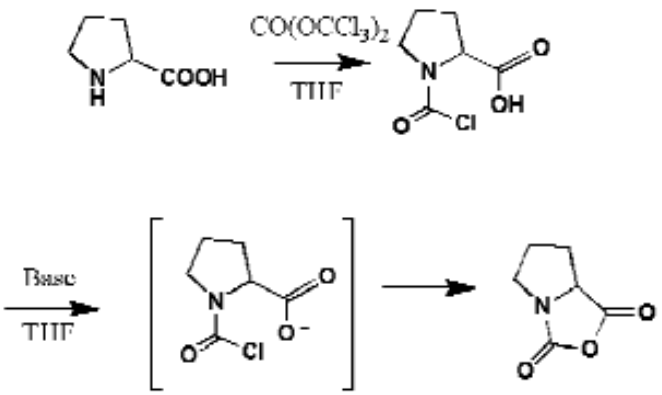

***Figure 5**. Synthesis of Proline NCA.*

We use evolutionary algorithms to generate potential peptide ligands of a given protein by minimising the docking energy between the candidate peptide ligand and a user-defined area of the target protein surface, or *surface patch*. To achieve this goal, an algorithm must address two main tasks. First, this high-dimensional chemical space must be examined using a competent search method. Second, the search space (*ie*, the set of all algorithmically treatable molecules) must be divided into regions of higher and lower quality to allow the prediction of desired properties.

The software we have developed has been successfully tested on the design of ligands for the proteins prolyl oligopeptidase, p53, and DNA gyrase.

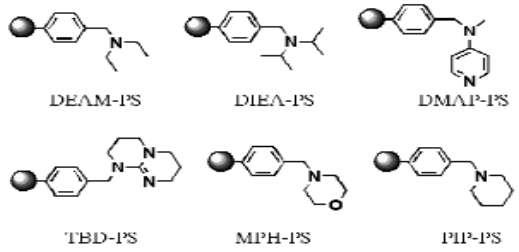

***Figure 6**. Polymer-bound tertiary amines used for the preparation of Proline NCA.*

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RESEARCH NETWORKS AND GRANTS

Aplicación de la RMN a la determinación y caracterización dinámica de estructuras de proteínas, a la identificación de ligandos y a la caracterización de los correspondientes complejos
GEN2003-20642-C09-04
Ministerio Ciencia y Tecnología: 2004-2007
Principal Investigator: Ernest Giralt

NANOFAR-Utilización de péptidos para la vectorización intracelular de nanopartículas
NAN2004-09159-C04-02
Ministerio Educación y Ciencia: 2005-2008
Principal Investigator: Ernest Giralt

Estudios estructurales y dinámicos de especies oligoméricas y fibrilares de Beta-Amiloide. Experimentos de intercambio protón/deuterio analizados por resonancia magnética nuclear (RMN) y espectrometría de masas (EM)
La Caixa: 2006-2008
Principal Investigator: Ernest Giralt

Diseño de ligandos peptídicos para el reconocimiento de superficies proteicas
BIO2005-00295
Ministerio Educación y Ciencia: 2005-2008
Principal Investigator: Ernest Giralt

Ajuts per potenciar i donar suport als grups de recerca - 013010, 2005SGR00663
Generalitat de Catalunya: 2005-2008
Principal Investigator: Ernest Giralt

Desarrollo de nuevos nanobiomateriales: Manipulación de la autoagregación y de la conformación de proteínas para reducir su toxicidad - Colaboración con la Universidad de Santiago (Chile)
Secretaría de Estado de Cooperación Internacional (AECI)
Ayudas para proyectos conjuntos de investigación, A/3388/05: 2006
Principal Investigator: Ernest Giralt

Síntesis de inhibidores peptídicos de la prolil oligopeptidasa (pop) ricos en prolina
PHB2005-0068-PC
Programa Hispano-Brasileño de Cooperación Interuniversitaria. Estancias de movilidad.
Ministerio de Educación y Ciencia: 2006-2007
Principal Investigator: Ernest Giralt

Diseño de "moléculas-espejo" de ligandos peptídicos: utilización de métodos de computación evolutiva
BIO2006-26119-E
Acciones Complementarias - Programa Explora Ingenio
Ministerio de Educación y Ciencia: 2006-2007
Principal Investigator: Ernest Giralt

Diseño, síntesis y estudio estructural de nuevos inhibidores de la dimerización de la proteasa del VIH
Exp 36606/06
FIPSE - Fundación para la investigación y la prevención del SIDA en España: 2006-2009
Principal Investigator: Ernest Giralt

COLLABORATIONS

Conformational analysis of somatostatin analogues
Jean Rivier (The Salk Institute, San Diego, USA)

Use of Silaproline in peptide research
Jean Martinez and Florine Cavelier (University of Montpellier, France)

Remote manipulation of protein aggregation
Marcelo Kogan (University of Chile, Santiago, Chile)

Mimicking discontinuous antigenic sites
David Andreu (University Pompeu Fabra, Barcelona, Spain)

Understanding resistance mechanisms against antimicrobial agents
Jordi Vila (Faculty of Medicine, University of Barcelona, Spain)

Cyclodepsipeptides as potential anticancer agents
Ricardo Pérez-Tomas (Bellvitge Hospital, University of Barcelona, Spain)

Structural studies in uteroglobin self-assembly
Ernesto Nicolás (Organic Chemistry Department, University of Barcelona, Spain)

Application of mass spectrometry to the study of protein-protein interactions
Eliandre Oliveira (Proteomics Platform, PCB, Spain)

Self penetrating proline-rich dendrimers and γ-peptides
Miriam Royo (Combinatorial Chemistry Platform, Parc Científic de Barcelona, Spain)

Synthesis and conformational analysis of cyclodepsipeptides from marine origin
Fernando Albericio (IRB Barcelona, Spain)

Mass-spectrometric analysis of post-transductionally modified proteins
Ferran Azorín (IRB Barcelona, Spain)

AWARDS

Susana Gordo (PhD Student)
Modifying p53 tetramerisation by designed calyx[4]arene compounds
Best oral presentation in the young investigator mini-symposium: Dr. Bert L Schram Award (29th European Peptide Symposium and the ESCOM Science Foundation), Gdansk-Poland, September 2006

Meritxell Teixidó
Novel approaches to study drug delivery to the brain
Best poster by a young scientist: Dr. Bert L Schram Award (29th European Peptide Symposium and the ESCOM Science Foundation), Gdansk-Poland, September 2006

Ernest Giralt's group, March 2006.

New synthetic methodologies and syntheses of biologically active molecules

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Antoni Riera Escalé

Our research activities focus on the synthesis of biologically active compounds for the various stages of drug development. Several of our projects address the development of basic synthetic methodology, with special emphasis on asymmetric synthesis. Others are devoted to the synthesis of compounds of known therapeutic interest, for which the emphasis is placed on the reliability, efficiency and scalability of the processes. Finally, other projects concern drug discovery and aim to prepare chemical libraries for biological screening.

Asymmetric catalysis. Basic synthetic methodologies

New strategies in the Pauson-Khand reaction

The Pauson-Khand reaction (PKR) is one of the most powerful reactions for the preparation of cyclopentanic compounds. The PKR is a cobalt-promoted or catalysed cycloaddition between an alkene and an alkyne with insertion of a carbon monoxide molecule to give a cyclopentenone. We use these cyclopentenones as starting materials for the synthesis of biologically active substances.

A ligand-based asymmetric version of the PKR must include a diastereoselective coordination step of the ligand with the cobalt carbonyl complex of the alkyne. Over the last year, we have studied how non-bonding interactions enhance the selectivity of this coordination. Finding the key for a high selectivity would open the door to development of a practical asymmetric version of the PKR.

With our hemi-labile P,S-ligands (CamPHOS and PuPHOS), we have disclosed how a non-classical hydrogen bond contact within the ligand and the dicobalt carbonyl substrate can dramatically increase selectivity (Solà *et al*, 2005; Solà *et al*, 2006). Although these results are significant due to the novelty of our approach, the use of the resulting diastereomerically pure complexes in the intermolecular PKR suffer several drawbacks. (See Figure 1.)

A new family of ligands for metal-catalysed reactions

To overcome the problems associated with the aforementioned approach, we designed a second generation of hemi-labile P,S-ligands. We synthesised these unprecedented, chiral non-racemic *N*-phosphinosulfinamides, and found that these ligands bound to dicobalthexacarbonyl complexes with high selectivity. The resulting complexes were successfully applied to the asymmetric PKR. The importance of these results led us to file a patent (Spanish application EP2006-02665) for these compounds and their use in PKR, before publishing the results. (See Figure 2.)

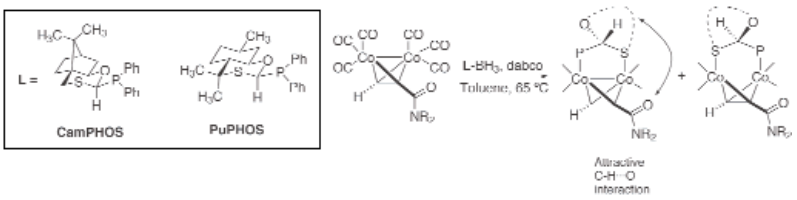

Figure 1. *Diastereoselective coordination of P,S-ligands directed by a non-classical hydrogen bond.*

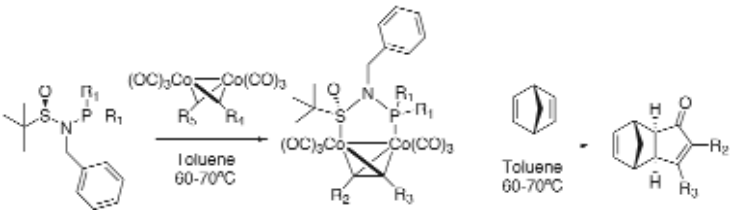

Figure 2. *New N-phosphinosulfinamide ligand and their use in enantioselective Pauson-Khand reactions.*

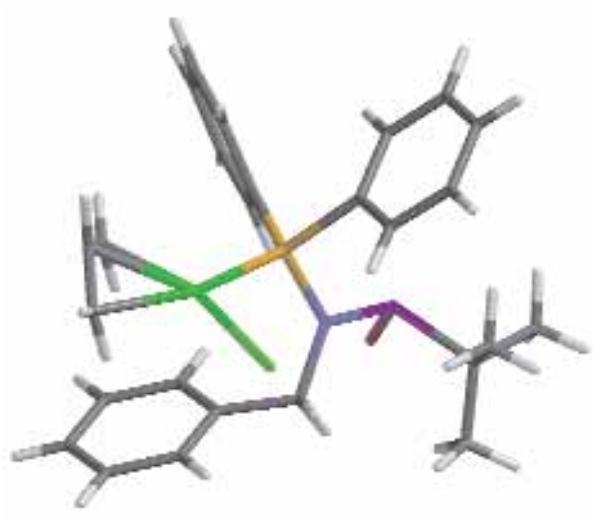

***Figure 3**. X-ray diffraction of a palladium complex of our N-phosphinosulfinamide ligand.*

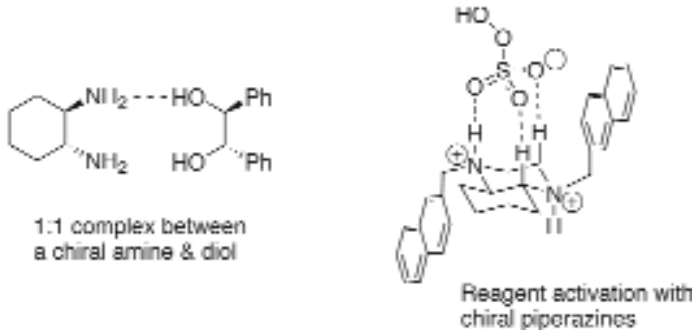

***Figure 4**. Complexes stabilized by hydrogen-bond interactions. An approach to new organocatalytic methods.*

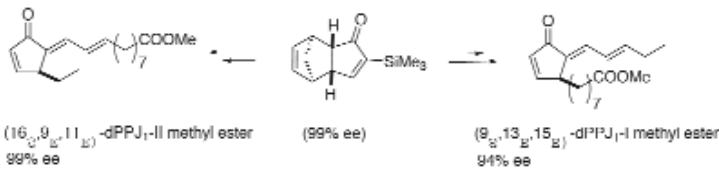

***Figure 5**. Syntheses of dehydrophytoprostanes from an intermolecular Pauson-Khand adduct.*

Application of the new *N*-phosphinosulfinamide ligands to other metal-catalysed processes is now being studied in our labs. The chemical nature of these ligands makes several coordination modes possible for a determined metal centre. Thus, they can act as monodentate ligands through phosphorous, or alternatively, as bidentate ligands through either phosphorous-oxygen or phosphorous-sulfur. To date, we have determined the structure of a palladium complex in which the ligand acts as a monodentate ligand. Interestingly, even in this case, these ligands provide a highly asymmetric environment around the metal centre. (See Figure 3.)

Assemblies of small molecules in asymmetric catalysis

We are currently studying catalytic reactions that do not use metal catalysts – also known as organocatalysis – with the aim of developing useful and clean asymmetric methodologies. One of our approaches is to form 1:1 complexes of organic molecules that could work in tandem as a catalyst. It is known that 1,2-diamines and 1,2-diols form 1:1 complexes in solution and in solid state. We are now exploring whether these 1:1 complexes can work as catalysts in the asymmetric Diels-Alder reaction via hydrogen bond activation of the substrates. We are also exploring the activation of these reagents through their complexing to chiral molecules via non-bonding interactions. In particular, we are studying the complex formation capacity of chiral piperazines and their corresponding salts. The ultimate goal is to activate oxidising agents such as oxone (sodium peroxomonosulfate), and to use these complexes in the asymmetric oxidation of pro-chiral substrates. (See Figure 4.)

Synthesis of biologically active compounds

Synthesis of five-membered ring compounds

As mentioned above, one of the best ways to prepare five-membered ring compounds is by the PKR. Prostaglandins are among the most important cyclopentanic compounds since they exhibit a wide variety of functions and biological activities. Some recently developed drugs are synthetic analogues of prostaglandins. Phytoprostanes are not as well-known. However, in the last few years, interest in their biological activity has increased considerably. These compounds have in common a cyclopentenone or a hydroxycyclopentenone ring, making them suitable substrates for synthesis by Pauson-Khand chemistry. In collaboration with Prof P Evans (Trinity College, Dublin), we have transformed the Pauson-Khand adduct of trimethylsilylethyne and norbornadiene into several dehydroisoprostanes (dPPJ1; see Figure 5). We are currently working on the transformation of Pauson-Khand adducts into prostaglandines

and phytoprostanes. We have also prepared a chemical library that has been tested in the search for specific inhibitors of beta-catenin.

Enantioselective synthesis of amino acids and amino alcohols

Amino alcohol fragments are present in many natural compounds such as aza-sugars, amino sugars, amino acids or sphingosines. We are currently working on the enantioselective synthesis of all these families of compounds, by developing convergent approaches based on the regioselective opening of epoxides, or sulfates, prepared by Sharpless asymmetric epoxidation (SAE) or dihydroxylations (AD).

We have recently published a methodology for the preparation of α-hydroxy-β-amino acids (Alonso *et al*, 2005), which has been applied to a practical, scalable synthesis of (2R, 3R)-2-amino-3-hydroxy-3-cyclohexylpropanoic acid, a key component of the anti-inflammatory and HIV antagonist drug ONO-4128 (Alonso *et al*, 2005; see Figure 6.)

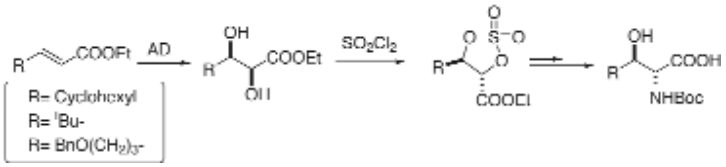

***Figure 6**. Enantioselective synthesis of β-hydroxy-α-amino acids.*

One of our most useful approaches for the preparation of cyclic compounds is based on the use of unsaturated epoxides to take advantage of the ring-closing metathesis (RCM) reaction. This year we have described the enantioselective preparation of all stereoisomers of 6-amino-cyclohex-3-ene-1,2-diols (4-deoxy-3-conduramines), key building blocks for the syntheses of a large range of natural products. Moreover, diastereoselective dihydroxylation of the compounds provided a new family of aminocyclitols (deoxyinosamines; Alegret *et al*, 2006). These compounds have been tested as potential β-catenin inhibitors, as explained below. (See Figure 7.)

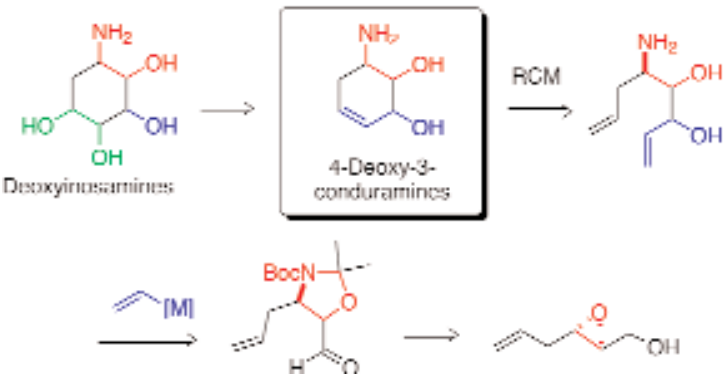

***Figure 7**. Synthetic approach to deoxyconduramines and aminocyclitols.*

Synthesis of new chemical libraries for drug-discovery

Synthesis of specific inhibitors of β-catenin

This project is being developed in collaboration with Prof Mireia Duñach (UAB) and Prof Antonio Garcia de Herreros (UPF).

The progressive accumulation of nuclear β-catenin that deregulates cellular proliferation, differentiation, and migration has been described as an initial event for the development of colonic tumourigenesis. In addition to its structural role in epithelial junctions, β-catenin activates TCF-4-mediated transcription of genes required for cell proliferation. (See Figure 8.)

Our hypothesis is that tumour progression can be arrested by blocking the aberrant transcription mediated by β-catenin. Our main goal is to identify of small molecules that specifically block β-catenin-

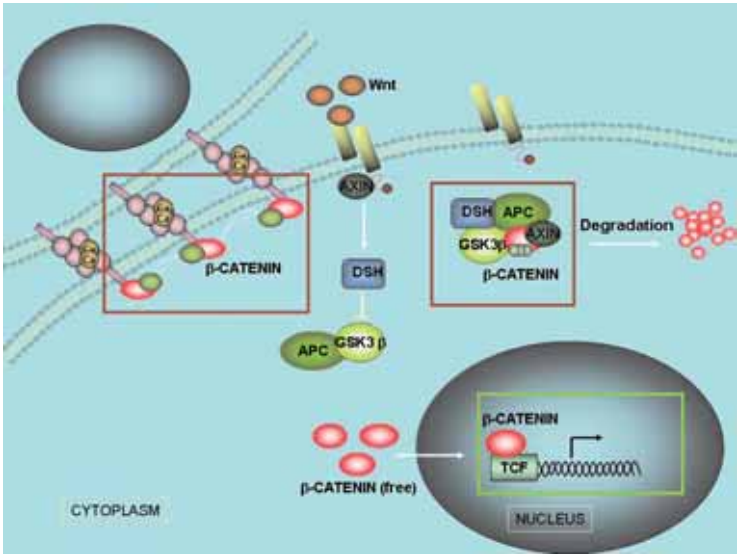

***Figure 8**. Double role of β-catenin: as a regulator of adherent junctions and as a transcriptional co-activator of genes involved in tumorigenesis.*

mediated transcription, which is essential for tumour development. These molecules could therefore be potentially active in colon tumour treatment.

We are searching for small molecules that do not alter the interaction of β-catenin with: a) factors involved in the establishment of adherens junctions (E-cadherin, α-catenin) and b) factors involved in the degradation complex (APC, axin). To this end, we have prepared several chemical libraries that have been screened in Duñach and Garcia de Herreros laboratories. We have tested a library based on the PKR, a library of amino alcohols, several non-natural amino acids derivatives and a peptidic library. The results are promising, although the work is still not ready for publication.

Synthesis of Somatostatin analogues

This year we have started a new project in collaboration with BCN-Peptides. A leader in API peptides, this company was recently awarded a CIDEM grant for this project. We have prepared and provided them with several adequately protected mesityl amino acids, which have been used in the synthesis of ten peptidic Somatostatin analogues. The biological activity of these new compounds is now being tested.

PUBLICATIONS

Alegret C, Benet-Buchholz J and Riera A (2006) Stereodivergent syntheses of conduramines and aminocyclitols. Organic Letters, 8:3069-3072

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RESEARCH NETWORKS AND GRANTS

Vers una nova família de catalitzadors per a processos reductius i síntesi asimètrica
Distinció de la Generalitat de Catalunya per a la promoció de la recerca Universitària. Categoria joves investigadors
DURSI (Generalitat de Catalunya): 2002-2006
Principal investigator: Xavier Verdaguer i Espaulella

Identificación de inhibidores específicos de la actividad transcripcional de la beta-catenina en cáncer de colon
Investigación biomédica, Convocatoria oncología, BM 05-68-0
Fundació La Caixa: 2006-2008
Project coordinator: Mireia Duñach Masjuan
Principal investigator of subproject: Antoni Riera Escale

Síntesis enantioselectiva de moléculas bioactivas mediante catálisis asimétrica: Reacciones de Pauson-Khand, organocatálisis y oxidaciones de Sharpless
Programme: NCTQ - Programa Nacional de ciencias y tecnologías químicas, CTQ2005-00623/BQU
DIGI - Dirección General de Investigación: 2006-2008
Entidades participantes: SPCT - Secretaría de estado de política científica y tecnológica / MEDU - Ministerio de Educación y Ciencia
Principal Investigator: Antoni Riera Escale

Identificació d'inhibidors específics de l'activitat transcripcional de la beta-catenina en la progressió tumoral
Ajuts econòmics a projectes de recerca sobre càncer, 050630/31/32
Fundació La Marató de TV3: 2006-2008
Project coordinator: Mireia Duñach Masjuan
Principal investigator of subproject: Antoni Riera Escale

OTHER FUNDING SOURCES

Three contract research projects with Enantia SL through the Fundació Bosch i Gimpera. Project numbers: FBG304021; FBG303967 and FBG303414

COLLABORATIONS

Synthesis of specific inhibitors of beta-catenin
Mireia Duñach (Universitat Autònoma de Barcelona, Spain)

Synthesis of specific inhibitors of beta-catenin
Antonio Garcia de Herreros (Universitat Pompeu Fabra, Spain)

Asymmetric catalysis
Miquel A Pericàs (The Institute of Chemical Research of Catalonia, Spain)

Synthesis and biological activity of phytoprostanes
Paul Evans (Trinity College, University of Dublin, Ireland)

Synthesis and biological activity of phytoprostanes
Martin Müller (Julius-von-Sachs-Institut of Biosciences, Universität Würzburg, Germany)

Synthesis of pharmaceutically active compounds
Enantia SL, Spain
Llorenç Rafecas, Alex Comely, Nicolas Tesson

Synthesis of Somatostatin analogues
BCN Peptides SL, Spain
Berta Ponsati, Jimena Fernández-Carneado, Marc Gomez

Antoni Riera Escalé's group, March 2006.

Peptidomimetics, bioactive heterocycles and enantiomeric recognition

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Màrius Rubiralta Alcañiz

The drug discovery process relies on the preparation of key bioactive compounds in pure form. Therefore, synthetic and purification technologies are critical for the development of this kind of project. Exploration in new reactivity pathways dealing with heterocycles (structures frequently found in drugs), new types of peptide-like molecules and the efficient separation of enantiomers constitute methodological tools in the first steps of drug discovery. Our main objective is to obtain new molecules to be applied in the biomedical field. To this end, our approach is based on heterocyclic and peptide synthesis. Our main therapeutic targets are cancer, AIDS, and infectious processes. Using several chromatographic and related technologies, we also study chiral molecules as a tool in the separation of enantiomers and other products of high added-value. We focus on the development of new synthetic pathways for the rapid and efficient synthesis of heterocyclic systems. In this regard, we explore new multi-component reactions using nitrogen and oxygen heterocycles.

Conformationally constrained peptidomimetics

We select known peptides of structural and bioactive interest to be used as models, and we synthesise small libraries of constrained derivatives designed by pharmaco-modulation. Once purified and fully characterised, the new molecules are submitted to structure-activity relationship (SAR) studies in order to improve the lead compounds. We are currently working on four peptide models of relevance in several pharmacological fields. However, the synthetic methods, the structural studies, and the biological approach are similar. In the case of antibiotics acting on the prokaryote cell membrane, structural studies have shown that some of the compounds aggregate, a characteristic required for the activity. The first collection of compounds has been sent to the Hospital Clínic in Barcelona for biological activity determination (MIC determination), and we are starting assays on model liposome membranes (López *et al*, submitted). In the second line of research, on inhibitors of the dimerisation of HIV1-PR, libraries have been synthesised by combining solid phase synthesis (SPS) and synthesis in solution (SS), and the compounds have been tested *in vitro* and *in vivo*. Most of the compounds inhibit HIV1-PR activity, and our calculations indicate a dissociative behaviour. However, only one compound showed some activity on cells resistant to HIV1-PR, which indicates that maybe the mode of action may not be that expected (Pinyol *et al*, submitted). Additional research efforts have addressed the synthesis of the lactams used. Two other lines of research, pseudo-miraziridines and pseudo-melanotans, seek to identify anticancer agents. We have successfully synthesised lactams for

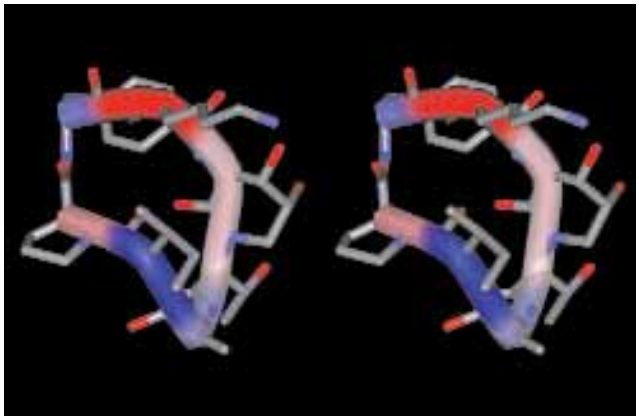

Figure 1. *Stereoview of* cyclo*(Phe-Pro-Lys-{Ser-Thr}-Ala-Ile-Pro). The lactam ring corresponding to {Ser-Leu} of this ψ-stylostatin is on the right side of the molecule. The Ile-Pro ω bond is in a* trans *conformation.*

the production of pseudo-miraziridines, and library production has started. All compounds will be subjected to biological assays. For pseudo-melanotans, we are currentlybuilding the lactams. Since the models have proved adequate, and the biological fields are hot subjects in drug development because of social health repercussions, we plan to continue searching for improved derivatives. Two PhD theses will be defended in a few months (Pinyol E and López P). (See Figure 1.)

Synthesis and reactivity of bioactive heterocycles

We study new reactivity pathways for relevant heterocycles (pyridines, indoles, oxygen-heterocycles, *etc*). Our main goal is to develop efficient synthetic methodologies for the preparation of bioactive compounds. Recently, we have started a project for the incorporation of N- and O-heterocycles in multi-component reactions (MCRs). In these processes, several molecules react to afford a single product, thereby increasing molecular diversity and exploratory power. These features make this approach very powerful in the development of SAR studies of drug-like compounds. We have designed new multicomponent procedures with pyridine derivatives, the most frequently found subunit in drugs, (substituted pyridines, quinolines and other azines, dihydroderivatives, *etc*) and oxa-heterocycles (5- and 6-membered rings, such as those present in carbohydrates and other bioactive compounds). The MCRs we have developed fall into two main classes: the Mannich-type processes (Povarov-like reactions) and the isonitrile-derived transformations (Ugi and Passerini reactions). These two classes have in common the search for structural diversity, selectivity and the practical character of the protocols to be developed. In this regard, we have reported the feasibility of using dihydropyridines (NADH analogues) as the azine components in MCRs (Carranco *et al*, 2005). The extension to oxygen heterocycles included a 4-component reaction which allowed the preparation of adducts derived from cyclic enol ethers (including glycals), amines, aldehydes and nucleophilic reagents called terminators, such as water, alcohols and thiols (Jiménez *et al*, 2005). For the Passerini and Ugi processes, we have developed several interesting transformations of dihydropyridines and cyclic enol ethers, which allow the straightforward carbamoylations of these privileged structures (Masdeu *et al*, 2006; Williams *et al*, 2006). We are currently exploring the bioactivity of the scaffolds prepared in collaborations with the groups led by Antoni Riera (IRB Barcelona) targeting β-catenin antagonists, Ernest Giralt (IRB Barcelona) aiming at the identification of proline oligopeptidase inhibitors, and Pelayo Camps (University of Barcelona) on the rapid synthesis of designed acetylcholinesterase inhibitors with anti-Alzheimer properties. An overview of the structures available from the MCRs is shown in Figure 2.

Enantioselective molecular recognition

Our main objective is to study the enantioselective recognition mechanism and its application to the separation of enantiomers, both at the analytic and the preparative level. This line of research involves the use of low molecular weight chiral selectors (derived from enantiomerically pure alpha-amino acids) and chiral macromolecules (derived from polysaccharides). In this regard, during the last year and taking advantage of our previous experience in the derivatisation and manipulation of polysaccharide (cellulose, amylose and chitosan) derivatives broadly used in enantioselective HPLC, we have applied, for the first time, this kind of material to the separation of the enantiomers of several chiral drugs by Centrifugal Partition Chromatography (CPC) (Pérez and Minguillón 2006b; Pérez *et al*, 2006). CPC is a separation technique that involves two liquid phases which has only recently been successfully applied to enantioseparation (Pérez and Minguillón, 2006a; Delgado *et al*, 2005). Our interest is also focused on the preparation of materials for membrane separations. Recently, a collaboration with C Palet's group introduced us to membrane technology and since then diverse derivatised polymers have been prepared and studied using this technology (Gumi *et al*, 2005; Cano *et al*, 2006). Moreover, diverse chiral selectors derived from L-proline have been used as carriers in liquid membranes (Gutiérrez *et al*, 2006). The enantiomeric transport observed using this set-up is close-

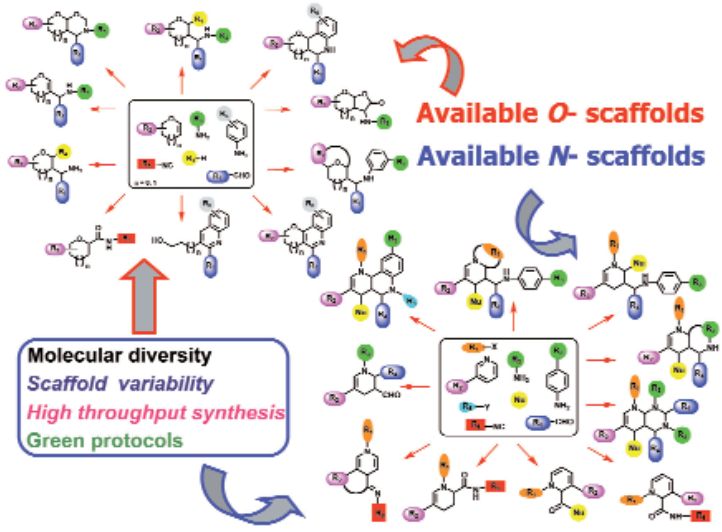

***Figure 2**. Available scaffolds from the MCRs with O- and N-heterocyles.*

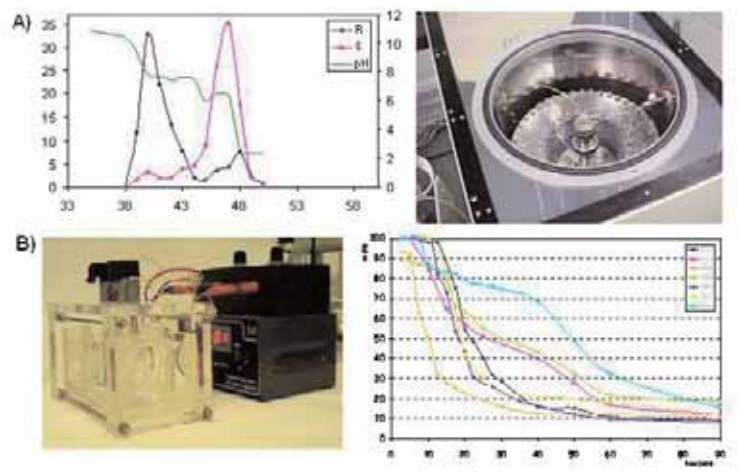

***Figure 3**. A) (left) Elution profile for the separation of pindolol enantiomers using a centrifugal partition chromatography device (right). B) (right) evolution of enantiomeric excess in the enantioselective transport of N-(3,5-dinitrobenzoyl)-(R)-phenylalamine through membranes containing L-proline derivatives as chiral selectors.*

ly related to the enantioseparation that occurs when using CPC. Both are biphasic liquid systems that are easy scalable to preparative/industrial dimensions, which accounts for the relevance of the preliminary results obtained. In parallel, we are also involved in the search for new chiral selectors. We have recently prepared and tested new polyproline-derived chiral selectors in enantioselective HPLC (Sancho *et al*, 2006). The resulting chiral stationary phases showed outstanding loading capacities (amount of racemate that can be processed in a single run). This property is of particular interest for preparative applications. As for polysaccharide derivatives, the adoption of helical conformations for this kind of chiral selector allows the presence of multiple identical recognition microenvironments, thereby explaining the high loadability characteristics of the two materials. At present, we are also addressing the enantiorecognition phenomenon from a more fundamental point of view. Thus, the chiral selector/enantiomers association is examined using several appropriate techniques available in our lab. Diverse NMR techniques, titration experiments, large-small molecule saturation transfer difference (STD) experiments among others, are in course. (See Figure 3.)

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Pérez E and Minguillón C (2006a) Countercurrent chromatography in the separation of enantiomers. In "Chiral separation techniques: a practical approach" (Subramanian G, Ed), Wiley-VCH, Weinheim

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Pérez E, Santos MJ and Minguillón CJ (2006) Application of cellulose and amylose arylcarbamates as

chiral selectors in counter-current chromatography. Chromatogr A, 1107:165-174

Sancho R, Pérez AM and Minguillón C (2006) A (4R)-hydroxy-L-proline-derived chiral scaffold and its oligomers as chiral selectors in liquid chromatography chiral stationary phases for enantioseparation. J Sep Sci, 29:905-914

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RESEARCH NETWORKS AND GRANTS

Xarxa de Química Médica
Ref: 2004XT-00011
CIRIT: 2005 - present
Project Coordinator: Rafael Franco

Xarxa de Bioinformática
Ref: 2003XT-00091
CIRIT: 2004 - present
Project Coordinator: Modesto Orozco

Xarxa de Prod. Naturales
Ref: 2004XT-00091
CIRIT: 2005 - present
Project Coordinator: Carlos Codina

Desenvolupament de sistemes policíclics nitrogenats d'interès biològic (Marius Rubiralta and Anna Diez)
Ref: 2005SGR-00158
CIRIT: 2006-2008
Project Coordinator: Ermitas Alcalde

Química combinatoria per al desenvolupament de nous compostos (Rodolfo Lavilla)
Ref: 2005SGR 00662
CIRIT: 2006-2008
Project Coordinator: Fernando Albericio

Grup de Química Farmacèutica (Cristina Minguillón)
Ref: 2005SGR00180
CIRIT: 2006-2008
Project Coordinator: Pelayo Camps

Combiestrategias para el descubrimiento de nuevos fármacos peptídicos y/o heterociclos (Rodolfo Lavilla)
Ref: BQU2003-00089
MCYT: 2004-2006
Project Coordinator: Fernando Albericio

Separación preparativa de enantiómeros: Cromatografía en contracorriente, membranas enantioselectivas y aplicación de la resonancia magnética nuclear al diseño de selectores quirales
Ref: PPQ2003-00970
MCYT: 2004-2006
Project Coordinator: Cristina Minguillón

Diseño, síntesis y evaluación biológica de peptidomiméticos y de pseudopéptidos de conformación restringida
Ref: CTQ2004 -01757/BQU
MCYT: 2005-2007
Project Coordinator: Anna Diez

OTHER FUNDING SOURCES

Síntesis de building blocks
Ref: FBG 303583
Fundació Bosch i Gimpera (Research project funded by Lab Esteve SA): 2005 - present
Project Coordinator: Anna Diez

Building blocks de interés para el proyecto de quimiocinas
Open project-account. Ref. FBG 302256
Fundació Bosch Gimpera: 2002 - present
Project Coordinator: Rodolfo Lavilla

Building blocks para lead finding
Fundació Parc Científic de Barcelona (Research project funded by Almirall Prodesfarma), APF 004: 2005-2006.
Project Coordinator: Rodolfo Lavilla

Assessorament i investigació aplicada en el camp de la química farmacéutica i orgánica
Open project-account. Ref: FBG 100027
Fundació Bosch Gimpera: 2002 - present
Project Coordinator: Cristina Minguillón

COLLABORATIONS

Activity assays of anti- HIV1 compounds
José Esté (Hospital Universitari Trias i Pujol, Barcelona, Spain)
Coordinator: Anna Diez

Activity assays of antibiotics
Jordi Vila (Hospital Clínic Provincial, Spain)
Coordinator: Anna Diez

Development of new inhibitors for acetylcholinesterase
Pelayo Camps (Department de Farmacologia i Química Terapèutica, Universitat de Barcelona, Spain)
Coordinator: Rodolfo Lavilla

Magdalena Grifoll (Department de Microbiologia, Universitat de Barcelona, Spain)
Identification of metabolites derived from polyaromatic hydrocarbons
Coordinator: Cristina Minguillón

Preparation of polysulfone derivatives to be used in membranes for advanced separations
Cristina Palet (Department de Química - Química Analítica, Universidad Autónoma de Barcelona, Spain)
Coordinator: Cristina Minguillón

Preparative enantiomer separation of several new acetylcolinesterase inhibitors
Pelayo Camps (Department de Farmacologia i Química Terapèutica, Universitat de Barcelona, Spain)
Coordinator: Cristina Minguillón

Purification of derivatised carbon nanotubes by CPC
Maurizio Prato (Department of Pharmaceutical Sciences, University of Trieste, Italy)
Coordinator: Cristina Minguillón

Synthesis and structure-activity relationships of HIV1-PR dimerisation inhibitors
Ernest Giralt (IRB Barcelona, Spain)
Coordinator: Anna Diez

Synthetic methodology. Synthesis of bioactive compounds. Nanotechnology
Fernando Albericio (IRB Barcelona, Spain)
Coordinator: Rodolfo Lavilla

Screening of libraries for catenine antagonists
Antoni Riera (IRB Barcelona, Spain)
Coordinator: Rodolfo Lavilla

Synthesis and structure-activity relationships of proline oligopeptidase inhibitors
Ernest Giralt (IRB Barcelona, Spain)
Coordinator: Rodolfo Lavilla

Research agreement
Enantia SA, Spain
Coordinator: Anna Diez

Research agreement
Procter & Gamble, Belgium
Coordinator: Anna Diez

Màrius Rubiralta's group, March 2006.

Oncology Programme

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Elena Sancho

MetLab

Wnt signalling, Eph/ephrin receptors and colorectal cancer

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Eduard Batlle

Colorectal Cancer (CRC) is one of the leading causes of death by cancer worldwide, killing around 400,000 people each year. Most colorectal tumours develop as benign lesions but a small proportion progress to more malignant stages when the appropriate alterations in oncogenes and tumour suppressor genes occur. The final and deadliest step in CRC progression is the metastatic dissemination of colorectal cancer cells to other organs, mainly the liver. Our lab studies the initiation of CRC and its progression from the early stages to the formation of aggressive tumours. We develop and examine cell and animal models that mimic this devastating disease. The ultimate goal is to obtain information that allows us to design new therapeutic and diagnostic tools.

Colorectal Cancer initiation and Wnt signalling
Around 70 % of all CRCs show homozygous inactivation of the Adenomatous Polyposi Coli (APC) tumour suppressor gene. This genetic alteration results in the activation of the Wnt signalling pathway and the constitutive transcription by the β-catenin/Tcf complex. Loss of APC function is present throughout the sequence of intestinal carcinogenesis (*ie*, from benign adenomas to fully malignant CRC and metastasis). Even the earliest precursors of colorectal tumours, the so-called dysplastic crypts, show mutational activation of the Wnt pathway. In mice, activating mutations in Wnt signalling pathway components lead to the formation of dysplastic crypts and benign adenomas similar to the preneoplastic lesions developed by humans. Together, these observations have led to the notion that constitutive transcription by the β-catenin/Tcf complex is the key step for initiation of CRC (reviewed in Sancho *et al*, 2003; Sancho *et al*, 2004)

Given the relevance of Wnt signalling for CRC cancer, we undertook the identification of the gene programme targeted by β-catenin and Tcf in the intestine. We identified a set of 100 genes which expression was driven by the β-catenin/Tcf complex in CRC cells. We showed that this genetic programme is also expressed by normal progenitor and stem cells of the intestinal epithelium, which receive physiological Wnt signals. Since this seminal finding, a main objective of our research has been to decipher the instructions given by β-catenin/Tcf to intestinal progenitors and CRC cells. Our and other laboratories have shown that β-catenin/Tcf appears to dictate three different sets of instructions that collectively regulate the biology of normal and transformed intestinal cells.

The core module of instructions enforces the undifferentiated-proliferative phenotype of progenitor crypt cells. Mice genetically manipulated to block β-catenin/Tcf activity in the intestine lack proliferative progenitors (Korinek *et al*, 1998; Pinto *et al*, 2003). Conversely, APC deficiency in the intestinal epithelium leads to an enormous amplification of the progenitor compartment at expenses of the differentiated compartment (Sansom *et al*, 2004). This core set of instructions also determines the proliferative-undifferentiated phenotype of CRC cells.

The second module of the β-catenin/Tcf programme is necessary for Paneth cell maturation (van Es *et al*, 2005; Andreu *et al*, 2005). Paneth cells are a secretory cell type localised close to the bottom of the crypts in the small intestine that display prominent nuclear β–catenin localisation.

EphB receptors and cell positioning in the crypts
The third module of instructions controls the compartmentalisation of epithelial cells along the crypt axis and regulates their ordered migration (Batlle *et al*, 2002). The main effectors of this function are the β-catenin/Tcf targets EphB2 and EphB3, two members of the Eph family of receptors. Eph receptors comprise the largest subgroup of receptor tyrosine kinases characterised for binding to membrane tethered ligands known as ephrins (Pasquale, 2005). Eph receptors act as a pathfinding clues for migrating cells or in the establishment of boundaries between different cell populations during embryonic development (Pasquale, 2005). This function has been associ-

ated with their ability to provoke cell repulsion upon activation by ephrin ligands. In the intestine, β-catenin/Tcf drives the expression of EphB2 and EphB3 in epithelial cells located at the crypt base, *ie*, stem cells and their immediate descendants as well as Paneth cells. We demonstrated that EphB2/EphB3 double mutant mice show defects in cell positioning in the crypts that include loss of the boundary between the proliferative and differentiated compartments, abnormal migration of precursor cells along the crypt axis and lack of compartmentalisation of Paneth cells at the bottom of the crypts (Batlle *et al*, 2002).

In our laboratory at the IRB we are trying to understand the molecular basis of cell positioning mediated by EphB receptors in the intestinal epithelium. We have recently generated an *in vitro* model that mimics EphB-ephrinB interactions by epithelial cells. We took advantage of the fact that several cell lines do not express EphB or ephrinB molecules to generate two populations of the same cell line that express either EphB receptors (plus GFP) or ephrinB ligands (plus RFP). Co-culture of EphB and ephrinB-expressing cells resulted in cell-cell contact activation of EphB-ephrinB bi-directional signalling. Analysis of cell dynamics in this *in vitro* model has revealed that EphB signalling induces repulsion, restricts cell intermingling and compartmentalises the growth of epithelial intestinal cells (Figure 1, Palomo S, Cortina S, Gallego L, Humà M, Jonkheer S, Soriano P, Sancho E and Batlle E. EphB receptors suppress colorectal cancer by compartmentalising tumour cells, submitted). We are currently using this model to identify and dissect the functions of EphB downstream signalling.

EphB receptors and colorectal cancer progression

We initially suspected the role of EphB receptors as suppressors of CRC progression after analysing the β-catenin/Tcf target gene programme in a collection of human CRC samples at different stages of malignancy (Batlle *et al*, 2005). Dysplastic crypts and small adenomas retained expression of most β-catenin/Tcf targets present in crypt progenitors pinpointing a common tumour initiation mechanism through mutational activation of the Wnt signalling pathway. These initial lesions showed homogenous EphB2, EphB3 and EphB4 expression in all cells at equivalent levels to that of normal crypt progenitors. Strikingly, the majority of colorectal carcinomas contained more than 50% EphB receptor negative cells despite evident nuclear β-catenin localisation. (See Figure 2.)

Does loss of EphB expression confer any advantages to CRC cells? The results of our genetic experiments were unequivocal. We engineered mice where the APCmin mutation was placed in a genetic background with low EphB activity. $APC^{min/+}$ mice develop benign intestinal lesions such as dysplastic crypts and adenomas as a result of constitutive activation of the Wnt signalling pathway. In the absence of EphB activity, tumour progression in the large intestine of $APC^{min/+}$ mice is strongly accelerated resulting in the development of aggressive colorectal adenocarcinomas. Therefore, while constitutive activation of the Wnt signalling pathway is required for the initiation of tumourigenesis (transition from normal epithelium to early adenoma stage), not all the instructions codified within the β-catenin/Tcf crypt progenitor programme promote tumourigenesis. Rather, the module that specifies cell positioning seems to block tumour progression beyond the earlier stages.

Tumour compartmentalisation: a new mechanism of tumour suppression?

At the onset of tumourigenesis, APC mutant-cells are confined to the epithelium within the so-called dysplastic crypts. These tumour-founder cells expand laterally and repopulate the surrounding crypts with their mutant descendants. It is during this initial phase that $EphB_+$ tumour cells are continuously in contact with normal epithelial cells expressing ephrinB ligands. An attractive hypothesis that we are currently testing is that tumour cells are forced to respect the boundaries imposed by EphB-ephrinB interactions much like normal progenitors and paneth cells are compartmentalised in the healthy tissue. Silencing of EphB receptors would generate a subset of tumour cells with unrestricted capacity for repopulating the epithelium. Alternatively, EphB receptors could also suppress tumour progression in more advanced stages. Thus, a fundamental issue is to identify the $ephrinB^+$ territories that restrict the colonisation of $EphB^+$ tumour cells during CRC progression. We have analysed the expression patterns of all ephrin ligands in a panel of intestinal tumour samples from patients. We have identified four potential sites of ephrinB-EphB interactions; the normal epithelium, mesenchyme, blood vessels and

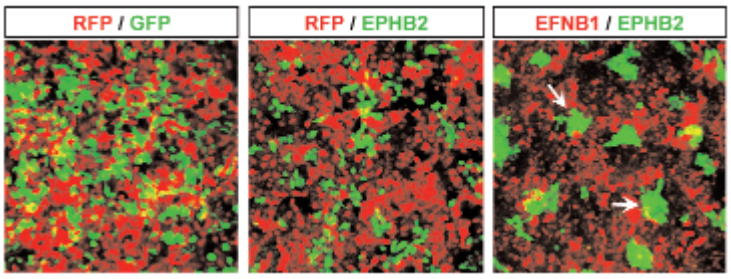

Figure 1. Examples of co-cultures of epithelial cells infected with lentivirus bearing GFP, EphB2-GFP or ephrinB1-RFP cDNAs. A dramatic cell sorting and compartmentalisation occurs in EphB-GFP/ephrinB1-RFP co-cultures (arrows).

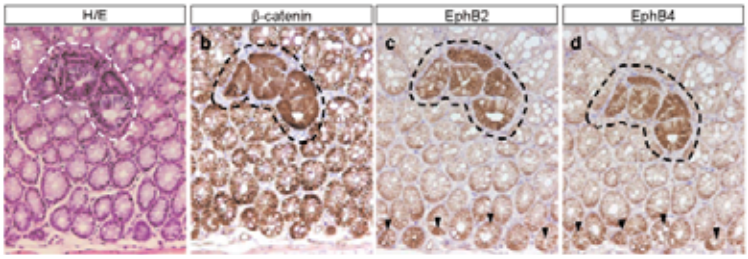

Figure 2. An early colorectal adenoma (dashed line) showing prominent nuclear β-catenin (b) accumulation and expression of EphB receptors (c and d). The arrows indicate normal crypt progenitor cells which also stain positive for EphB receptors.

lymph nodes. We are studying the relevance of the different EphB-ephrinB interactions for tumour progression by generating *in vitro* and *in vivo* models that mimic these scenarios.

The mechanism of EphB silencing during tumour progression

While genetic alterations or epigenetic silencing in individual EphB genes have been identified in a fraction of CRCs, our observations indicate that EphB2, EphB3 and EphB4 are co-ordinately silenced in the majority of CRC samples (Batlle *et al*, 2005). Thus, it is unlikely that mutations, LOH or methylation account for the coordinated silencing of all three EphB genes during CRC progression. In the great majority of cancers downregulation of EphB receptors occurs at the mRNA level (Batlle *et al*, 2005). EphB expression is fully dependent on β-catenin/Tcf activity yet many CRCs and cell lines downregulate EphB levels despite constitutive activation of the Wnt signalling pathway. Together, these observations point to a common mechanism of transcriptional silencing of EphB genes that acts in a dominant fashion over β-catenin/Tcf activation. By profiling $EphB^+$ and $EphB^-$ CRC cells, we have identified several genes that are bona-fide regulators of EphB expression during CRC progression. We have obtained evidences that these genes represent a complex genetic network regulating the adenoma-carcinoma transition. We are currently addressing the specific roles of these candidate genes in the acquisition of malignancy.

EphB receptors as markers of malignancy

Epithelial tumour cells that compose benign colorectal lesions such as small adenomas stain homogenously positive for EphB2, EphB3 and EphB4 receptors. Lesions at the adenoma-carcinoma transition (*ie*, large adenomas) contain clusters of tumour cells negative for EphB receptors expression. In carcinomas, the $EphB6^-$ population occupies the majority (>50%) of the tumour mass (Batlle *et al*, 2005). The increase in frequency of $EphB^-$ tumour cells is strongly associated with malignancy. $EphB^-$ cells are preferentially associated with poorly differentiated areas and invasion fronts of carcinomas while $EphB^+$ cells are organised in well-differentiated regions. As adenomas represent the benign precursors of carcinomas and tumours of higher grade often behave more aggressively than low grade ones, our observations implied that silencing of EphB expression has occurred in a subset of tumour cells concomitantly with the acquisition of malignancy. Our laboratory is currently testing whether the ratio of $EphB^+$ and $EphB^-$ cells in a tumour predicts clinical outcome of CRC patients. Also, we are using the gene expression signature obtained from $EphB^+$ and $EphB^-$ CRC cells to classify tumour samples from patients.

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Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR and Winton DJ (2004) Loss of Apc *in vivo* immediately perturbs Wnt signalling, differentiation, and migration. Genes Dev, 18:1385-1390

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RESEARCH NETWORKS AND GRANTS

Characterization of the mechanism controlling Intestinal Stem Cell specification and positioning. Use of intestinal Stem cells for tissue regeneration.
European Union FP6 - MERG-CT-2004-006329:2005
Principal Investigator: Eduard Batlle

Papel de los Receptores EPHB en el posicionamiento de las células epiteliales y en el cancer colorectal (The role of EphB receptors in intestinal cell positioning and colorectal cancer)
Ministerio de Eduación y Ciencia (SAF2005-04981):2006-2008
Principal Investigator: Eduard Batlle

Start-up grant for emergent research groups
Agencia de Gestió d'Ajuts Universitaris i de Recerca (Catalan Government) (2005SGR 00775): 2006-2009
Principal Investigators: Eduard Batlle Gómez and Elena Sancho Suils

Variations in the genetic program under the control of β-catenin/Tcf during colorectal cancer progression
Fundación La Caixa: 2007-2009
Principal Investigators: Eduard Batlle Gómez and Elena Sancho Suils

COLLABORATIONS

Hans Clevers (Hubrecht Laboratorium, Utrecht, Netherlands)

Giancarlo Marra (Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland)

Francisco X Real (Institut de Investigació Mèdica, Barcelona, Spain)

Elena Sancho (IRB Barcelona, Spain)

AWARDS

Debiopharm Life Sciences Award for Outstanding Research in Oncology, 2006
Ecole Polytechnique Fédérale de Lausanne, Switzerland

Eduard Batlle's group, March 2006.

Molecular mechanisms involved in colorectal cancer initiation and progression

Principal Investigator
Elena Sancho

Postdoctoral Fellow
Agata M d'Agostino

PhD Students
Elisa Espinet
Neda Motamedi-Shad

Research Assistant
Sergio Palomo

Technician
Mireia Humà

Elena Sancho

Last year alone, more than half a million people worldwide died of Colorectal Cancer (CRC), making CRC the second cause of death by cancer. Most sporadic colorectal cancers arise from adenomas that initially are benign and occur frequently: approximately 50% of the Western population develops an adenoma by the age of 70. However, the development of a full-blown malignant colorectal tumour is a progressive process that often takes several years. During this period, the progression of the disease appears to follow a precise series of molecular events, requiring the accumulation of mutations in proto-oncogenes and tumour suppressor genes in these initially benign lesions. Access to specimens of CRC at different stages of the malignancy has allowed the analysis of the molecular alterations most frequently associated with each step of the disease (reviewed in Sancho *et al*, 2004). Our research aims to decipher the molecular instructions that underlie the signalling pathways that are altered in CRC and are responsible for the initiation and the progression of the disease.

WNT signalling and the initiation of CRC

Around 70% of sporadic colorectal tumours show biallelic inactivation of the APC gene (*Adenomatous Polyposis Coli*). A high percentage of remaining tumours show activating mutations in β-catenin or axin. These molecules are all components of the Wnt signalling pathway. Activating mutations of the Wnt signalling pathway are the only known genetic alterations present in early premalignant lesions in the intestine, such as aberrant crypt foci and small adenomas. In various animal models, activating mutations in this pathway effectively initiate tumourigenesis in the intestine in a process characterised by the formation of displastic cripts and adenomas similar to those found in humans. Therefore, it is widely accepted that constitutive activation of Wnt signalling caused by mutations in components of the pathway is responsible for the initiation of CRC (reviewed in Sancho *et al*, 2004). (See Figure 1.)

Mutations in Wnt signalling components that lead to CRC result in the stabilisation and accumulation of β-catenin in the nucleus, and, as a result in increased transcriptional activation mediated by the β-catenin/TCF complex. Therefore, the transactivation of β-catenin/TCF target genes is believed to represent the primary transforming event in CRC. A few years ago we undertook the identification of the genetic programme driven by β-catenin and Tcf in CRC cells. We engineered CRC cell lines bearing activating mutations of the Wnt pathway, which allowed us to block the constitutive β-catenin/Tcf mediated transcription in an inducible manner (Van de Wetering *et al*, 2002).

Gene expression profile analysis of CRC tumour cells before and after blockage of β-catenin/TCF activity revealed a set of approximately 100 target genes. We have analysed the expression pattern of these mole-

Figure 1. Genetic alterations frequently associated with CRC tumour progression.

cules in tumours and confirmed that are consistently expressed in dysplastic crypts and adenomas. Strikingly, we found that the same genetic programme was expressed in normal non-transformed intestinal progenitors cells at the bottom of the crypts (Van de Wetering *et al*, 2002). (See Figure 2.)

These observations were unexpected since at the time it was believed that *de novo* activation of wnt signalling in tumour cells was the initial event triggering transformation. Our additional research efforts demonstrated the presence of β-catenin, and thus physiological wnt signalling, in the nucleus of a few cells at the bottom of the crypts where the intestinal progenitors reside. These findings led us to propose that in CRC the first step towards malignancy consists of the acquisition of a crypt progenitor-like phenotype (Van de Wetering *et al*, 2002). Our hypothesis has signified a milestone in the field and has completely changed views on the initiation of CRC.

Animal models in which Wnt signalling has been genetically manipulated support this notion. Mice deficient for Wnt signalling in the intestine, either through the expression of a transgene encoding the Wnt signalling inhibitor Dickkopf (DKK) or TCF4-deficient mice lack progenitors in crypts (Pinto *et al*, 2003; Korineck *et al*, 1998), whereas conditional deletion of the tumour suppressor APC leads to an abnormal expansion of the progenitor compartment of the crypts, at the expense of the compartment containing differentiated cells (Sansom *et al*, 2004). (See Figure 3.)

In addition, we have described that blockage of Wnt signalling in CRC cells leads to cell cycle arrest and differentiation, despite the presence of multiple other mutations in other pathways, indicating that β-catenin/TCF activity is the main mediator of CRC cell proliferation. Amongst the target genes under the control of β-catenin and TCF, we identified the oncogene c-myc as the critical molecule for proliferation of CRC cells through the repression of the cell cycle inhibitor $p21^{cip1/waf1}$ (Van de Wetering *et al*, 2002). Consistent with c-myc being an essential executor of β-catenin/TCF instructions in the maintenance of the progenitor proliferative phenotype, mice deficient for c-myc in the intestine show a rapid loss of progenitors in intestinal crypts (Muncan *et al*, 2006).

Having identified that the initial event triggering transformation is the blockage of founder tumour cells into a progenitor phenotype, our lab now seeks to identify differences between the true physiological progenitors and initial founder mutant cells. During this year we have started a project to identify tumour-specific molecular targets susceptible to being targeted by the pharmaceutical industry. These will be particularly useful for patients suffering Familial Adenomatous Polyposis (FAP). These patients inherit a mutation in APC and as a result of a loss of heterozygosity (LOH), they develop hundreds of polyps in the intestinal tract and therefore, are predisposed to the development of malignant CRC. Our studies are also oriented towards the identification of the nature of the founding CRC cell and the mechanisms by which it escapes cell renewal. These studies may shed additional light on specific pathways that may be targeted to block CRC progression. Likewise, in collaboration with Dr. Eduard Batlle's laboratory, we intend to identify the core set of instructions imposed by Wnt signalling mutations that remains unaltered throughout the carcinogenetic process. This will be addressed through systematic analysis of CRC samples at distinct stages of the disease. The results from this analysis will yield crucial data regarding molecular targets for CRC at all stages of the malignancy.

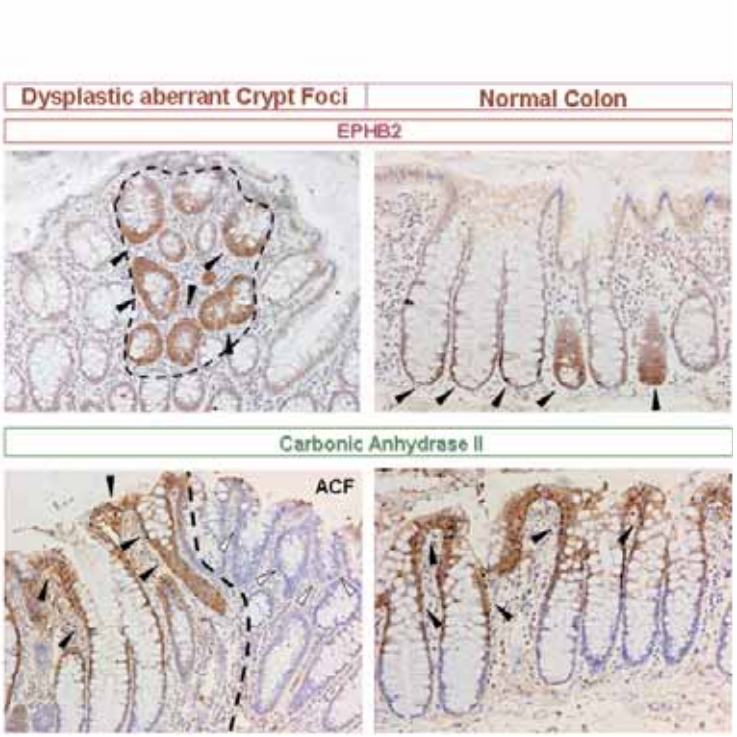

***Figure 2**. Expression pattern of EphB2 (A,B) and Carbonic Anhydrase II (C,D) in early tumour lesions. EphB2, an example of a target gene under the control of β-catenin/TCF, is expressed by tumour cells from displastic crypts (A, black arrows), but also by normal progenitor cells at the bottom of intestinal crypts (B, black arrows). In a complementary fashion, the differentiated marker carbonic anhydrase II is expressed only in the top half of the crypts and surface epithelium (C,D black arrows), and is absent from tumour lesions (C, white arrows). This expression pattern is observed for the β–catenin/Tcf target genetic programme identified so far.*

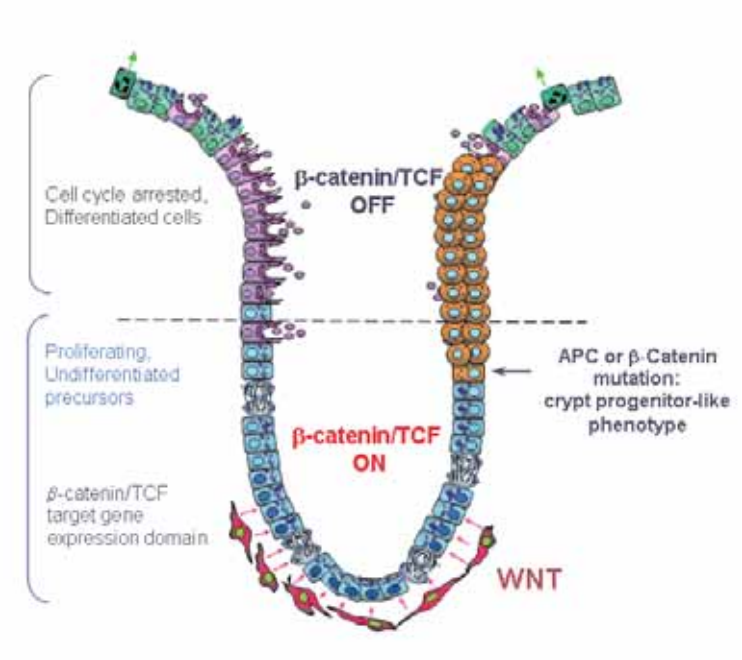

Figure 3. Schematic representation of a colon crypt in relation to wnt signalling and proposed model for the initiation of colorectal cancer. The proliferative compartment of intestinal crypts is maintained by the target gene program directed by β-catenin/Tcf in response to wnt signals. When these cells physiologically down-modulate β-catenin/Tcf activity, they cease proliferation and differentiate. Cells mutant in components of wnt signalling (APC, β-catenin, axin) become independent of these signals, exhibit constitutive activation by β-catenin/Tcf, and are blocked in a progenitor proliferative phenotype.

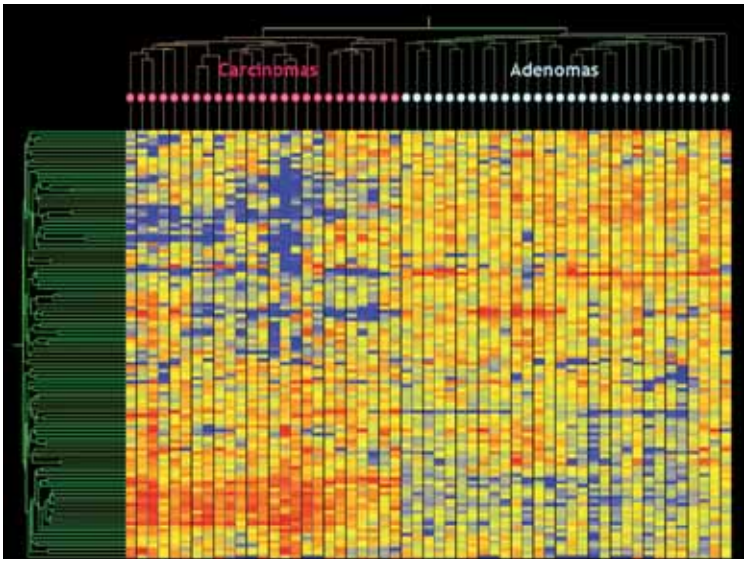

Figure 4. The TGF-β signature discriminates between adenomas and carcinomas. Unsupervised clustering analysis of a collection of tumours of known transcriptomes on the basis of target genes controlled by TGF-β signalling, clearly classifies adenomas and carcinomas in two separate branches.

TGF-β signalling during CRC progression

In recent years, some of the leading scientific teams, including ours, have pointed out that the emerging and progression of CRC can be explained by some concepts from the Darwinian evolution model. Under this view, the sequence of mutations acquired during CRC progression (Figure 1) can be understood as if colorectal tumours evolve through a series of bottlenecks or restriction points at which only those cell cells acquiring the correct mutational event expand and progress to the next stage of malignancy. Our research activities seek to determine how the acquisition of mutations in other signalling pathways may modulate the initial progenitor phenotype imposed by Wnt signalling in order to overcome the bottlenecks associated with CRC progression.

One of the most prevalent types of mutations found during CRC progression are those that inactivate the TGF-β signalling pathway (reviewed in Grady and Markowitz, 2003). Our lab currently focuses on the role of TGF-β signalling in CRC progression. The TGF-β pathway is involved in numerous processes in the development and homeostasis of adult tissues. TGF-β ligands activate the signalling pathway by binding to TGF-β receptor type II homodimers. Ligand-bound receptor II recruits TGF-β receptor I homodimers, which are subsequently transphoshorylated and thus activated by receptor type II. Phosphorylation of the intracellular mediators smads by activated receptor I allows dimer formation with smad-4 and translocation to the nucleus where the specific outcome of the signalling will depend on the cell type and the context of the cell itself (reviewed in Shi and Massagué, Cell 2003). Mutations found in CRC affect mainly TGF-β receptor type II and the intracellular smads, smad-2 and smad-4, abolishing transcriptional activation or repression mediated by TGF-β.

We are currently studying the transcriptional events controlled by TGF-β in CRC cells. We have already identified changes in approximately 500-genes in response to TGF-β. Unsupervised analysis of a collection of tumours of known transcriptomes on the basis of the TGF-β signature obtained in our laboratory perfectly discriminates adenomas from carcinomas, implying that these genes may contain the information that drives the adenoma/carcinoma transition. We are now dissecting this information in order to identify TGF-β genes that play an executive role in this transition. (See Figure 4.)

RECENT PUBLICATIONS

Batlle E, Beghtel H, JonkHeer S, Gregorieff A, van den Born M, Malats N, Sancho E, Boon E, Pawson T, Gallinger S, Pals S and Clevers H (2005) EphB activity suppresses colorectal cancer progression. Nature, 435:1126-1130

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RESEARCH NETWORKS AND GRANTS

β-catenin/TCF target gene programs driving intestinal stem cell maintenance, colorectal cancer initiation and progression
European Union FP6 - MERG-CT-2004-006357: 2005
Principal Investigator: Elena Sancho Suils

Molecular mechanisms involved in colorectal cancer initiation
Spanish Ministry for Education and Science (SAF2005-002170): 2006-2008
Principal Investigator: Elena Sancho Suils

Start-up grant for emergent research groups
Agencia de Gestió d'Ajuts Universitaris i de Recerca (2005SGR 00775): 2006-2009
Principal Investigators: Eduard Batlle Gómez and Elena Sancho Suils

Variations in the genetic program under the control of β-catenin/Tcf during colorectal cancer progression
Fundación La Caixa: 2007-2009
Principal Investigators: Eduard Batlle Gómez and Elena Sancho Suils

COLLABORATIONS

Giancarlo Marra (Institute of Molecular Cancer Research, Zurich, Switzerland)

Hans Clevers (Hubrecht Laboratory, Utrecht, Netherlands)

Elena Sancho's group, March 2006.

Tumoural Metastasis Laboratory (Metlab)

Managing Director
Roger Gomis

Adjunct Director, IRB Barcelona
Joan Massagué

PhD Students
Maria Tarragona Sunyer
Anna Arnal Estapé

Lab Manager
Marc Guiu Comadevall

Roger Gomis

Intricate signalling networks control cell division, differentiation, movement, organisation and death. Cancer cells disobey these signals during tumour progression and metastasis, which is the final step in 90% of all fatal solid tumours. Metastasis is therefore a grave public health problem and consequently a field of considerable pharmaceutical interest. A major research focus of our group is to identify and study the genes and functions that allow tumour cells to achieve metastatic colonisation of vital organs.

Our research focuses on the growth factors, signalling pathways, and gene expression programmes underlying cancer cell metastasis. We study the ways in which cancer cells evade tumour suppressor mechanisms and engage in metastatic behaviour. Focusing on a TGF-β cytostatic programme involving the transcriptional regulation of cell cycle inhibitors and growth-promoting factors, we are investigating how tumour cells evade these gene responses in order to pursue metastatic behaviour. By combining *in vivo* selection of human metastatic cells, transcriptomic profiling and functional testing, we identify genes that selectively mediate breast metastasis to specific organs. Gene transfer techniques and RNAi-mediated gene silencing are used to functionally validate candidate genes. We are encouraged by the recent validation of these findings in clinical samples. Several of these genes encode products that are susceptible to therapeutic targeting.

The Tumour Metastasis Laboratory (Metlab), part of IRB Barcelona's Oncology Programme focuses on the molecular mechanisms involved in metastasis. The activities in this research group began in June 2006.

Current research builds, in part, on recent progress in the analysis of the TGF-β cytostatic programme and its evasion in metastatic breast cancer. This line of research seeks to clarify the role of C/EBPβ transcription factor in the TGF-β cytostatic programme in epithelial cells. Recent results have provided a new approach to study the molecular mechanisms that regulate this programme. The TGF-β signalling process is based on the formation of a TGF-β-activated receptor complex that phosphorylates SMAD transcription factors, which in turn assemble molecular complexes that regulate the expression of target genes. Several of these gene responses act in concert to cause cell cycle arrest. This TGF-β cytostatic programme includes repression of the proliferation-promoting genes c-MYC and ld1, as well as induction the cyclin-dependent kinase (CDK) inhibitors p15INK4b and p21CIP1 (Massagué and Gomis, 2006). Repression of c-MYC and ld1 is mediated by a complex of SMAD with E2F4/5 and ATF3, respectively.

FoxO factors were identified as partners of TGF-β-activated SMAD3 in the induction of the CDK inhibitor, p21CIP1, in epithelial cells. In recent work, we have taken a genetic approach to identify other TGF-β target genes that are regulated by a common SMAD3/FoxO transcription complex. By using siRNA techniques coupled with gene expression microarray data analysis, 10 new genes whose TGF-β expression is induced by the same complex were identified (Gomis *et al*, 2006a). *p15INK4b* (Gomis *et al*, 2006a) stands out among these genes. Surprisingly, a detailed analysis of the *p15INK4b* promoter led to the identification of the role of C/EBPβ in *p15INK4b* induction by TGF-β (Gomis *et al*, 2006b).

Breast cancer cells are refractory to TGF-β-mediated growth arrest, which leads to further tumour progression and metastasis. The molecular characterisation of TGF-β-mediated cytostasis in keratinocytes has positioned C/EBPβ at the core of this response. Furthermore, deregulation of C/EBPβ mediates evasion of the TGF-β-induced cytostatic effects in metastatic breast cancer cells. We found that the transcription factor C/EBPβ is essential for not only the induction of the cell cycle inhibitor p15INK4b by a FoxO-Smad complex but also for the repression of c-MYC by an E2F4/5-Smad complex.

Interestingly, the p15INK4b and c-MYC gene responses, which are central to the TGF-β cytostatic pro-

gramme, are selectively missing in primary metastatic breast cancer cells from half of the patients with advanced-stage diseased that we analysed. Remarkably, this loss coincides with increased expression of the C/EBPβ inhibitory isoform LIP, which has been implicated in tumour progression. By normalising the LIP:LAP ratio, we restored these TGFβ cytostatic gene responses and growth inhibition in primary metastatic cells derived from patients. Building on this work, we will determine the mechanism by which LIP expression is deregulated in metastatic breast cancer cells.

Thus, using biochemical and molecular biology techniques, primary human breast cancer cell cultures and animal model studies, we examine the molecular mechanisms that lead to the deregulation of the C/EBPβ function and consequent loss of the TGF-β cytostatic response in cancer cells. In addition, our lab activities focus on extending these findings to other cell types in which the TGF-β cytostatic response is either permanently or temporally absent.

The second research project initiated in our lab seeks to identify gene groups that drive metastatic cells to one tissue or another. Particularly, we focus on metastatic suppressor genes and their functions in the metastatic process. Our initial studies are devoted to the group of metastatic suppressor genes required for breast to lung metastasis, identified in Dr. Joan Massagué's lab (Minn *et al*, 2005). For this purpose, we use the MDA-MB-231 breast cancer cell line model and its derivatives #4175 and #1833, which have a strong metastatic capacity to lung and bone. Furthermore, we are also screening new metastatic cell populations from pleural effusions derived from breast or lung cancer patients to identify new metastatic gene signatures. For this purpose, on the basis of collaborations with clinical and basic investigators at the Hospital Clínic, in Barcelona, and the Memorial Sloan-Kettering Cancer Center, in New York, the MetLab team has initiated the isolation of metastatic cells from pleural effusions derived from lung and breast cancer patients. These cells are labelled with the GFP-Luciferase-TK protein fusion and, once injected in mice, are visualised by bioluminescent techniques. From these metastatic cell populations, highly aggressive subpopulations with tropism to specific tissues will be isolated. These subpopulations will be used to identify and validate metastatic gene signatures by means of gene expression profile analyses and biochemical, cellular and molecular biology techniques.

PUBLICATIONS

Gomis RR, Alarcon C, He W, Wang Q, Seoane J, Lash A and Massagué J (2006) A FoxO-Smad synexpression group in human keratinocytes. Proc Natl Acad Sci USA, 103:12747-12752

Gomis RR, Alarcon C, Nadal C, Van Poznak C and Massagué J (2006) C/EBPβ at the core of the TGFβ cytostatic response and its evasion in metastatic breast cancer cells. Cancer Cell, 10:203-214

Massagué J and Gomis RR (2006) The logic of TGFβ signalling. FEBS Lett, 580:2811-2820

OTHER FUNDING

Mechanisms of metastasis
Fundación BBVA

COLLABORATIONS

Identification of genes involved in breast and lung cancer metastasis
Cristina Nadal (Servei D'Oncologia, Hospital Clínic Universitari de Barcelona, Spain)

MetLab, March 2006.