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MECHANISMS OF SIGNAL TRANSDUCTION

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ABSTRACTS

Liprin-α1 is an important player in integrin-mediated cell motility and invasion

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Liprin-α1 is a ubiquitously expressed member of the conserved Liprin-α family of scaffold proteins that includes four different isoforms in vertebrates. Liprin-α1 function in non-neuronal cells is poorly characterized, but its localization at focal adhesions makes it an interesting player in integrin-mediated cell motility. Cell migration is a dynamic process requiring the formation of lamellipodia and new adhesive sites, where integrins play a central role by linking the extracellular matrix to the actin cytoskeleton. We have identified a functional interaction between liprin and integrins by studying the function of liprin during cell spreading on fibronectin. Liprin overexpression positively influences spreading on fibronectin without affecting adhesion per se. These effects correlate with the strengthening of large actin-rich lamellipodia, and the relocalization of active β1-positive focal adhesions at the edge of spreading cells. On the other hand, liprin down regulation inhibits the formation of lamellipodia and cell spreading. A functional interplay between liprin and talin is also needed for efficient spreading. To test the proposed implication of liprin-α in tumor cell invasion, we have used human breast carcinoma MDA MB 231 cells to look at the effects of liprin depletion on the invasive potential of these cells *in vitro*. Modified Boyden chamber assays have shown that liprin is required for haptotactic migration and support the ability of these cells to invade matrigel matrices. Our results show that liprin-α1 is important for integrin-mediated migration during MDA MB 231 invasion, and indicate an important function of liprin in modulating the dynamic assembly of focal adhesions.

Constitutively active STAT3 synergizes with the ErbB-2/Neu oncogene resulting in increased invasiveness and motility of mammary tumors/tumor cells

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The transcriptional activator STAT3 is considered an oncogene, being constitutively active in many tumors where its inactivation induces growth arrest and apoptosis. However the molecular mechanisms involved are not fully understood. To investigate the role of STAT3 in mammary tumorigenesis, we intercrossed MMTV-Her2Neu transgenic mice (NeuT), which develop multifocal mammary adenocarcinomas at high multiplicity, with mice expressing a constitutively active form of STAT3 (STAT3C). NeuT;STAT3C mice develop faster growing, more invasive, less differentiated and less apoptotic tumors. Interestingly, tumor-derived cell lines from NeuT;STAT3C mice show increased invasive capacities. NeuT;STAT3C cells form more dynamic cell junctions and actin stress fibers. Accordingly, NeuT;STAT3C cells show a much higher metastatic potential in vivo. Microarrays analysis of tumor-derived cell lines revealed differential expression of Galectin3, Cten, Twist and Lypd3 between NeuT;STAT3C and NeuT;STAT3WT cells. We focused our attention on Cten, a peculiar member of the Tensins family. Tensins are cytoplasmic proteins that link the actin cytoskeleton to integrin-based adhesion sites. Although Cten lacks the N-terminal actin binding domain and could contribute to form more dynamic cell-matrix contacts. Cten silencing in NeuT;STAT3C results in significant inhibition of their migration potential and partially reverted the defects in cell-cell junctions. Moreover, Cten silenced cells adopted a more epithelial phenotype and displayed more continuous, enhanced cortical localization of epithelial markers. All together, these results suggest that the constitutive active STAT3 can synergize with the Neu oncogene promoting cell survival and triggering cytoskeleton and cell junctions reorganization, thus enhancing motility and invasion/metastases. Work is in progress to better characterize the specific roles of the STAT3 target genes involved.

Unacylated ghrelin recovers neovascularization in diabetes by targeting enos and redox signaling

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A relative excess of acylated ghrelin (AG), compared to the unacylated form (UAG), and an impaired circulating level of angiogenic cells (CAC) are common features in diabetes. In the present study we investigate the therapeutic potential of UAG in this setting. We demonstrate that UAG, by regulating the NADPH oxidase subunit Rac1, protects diabetic CAC from oxidative stress and from p53-mediated senescence like-growth arrest leading to an improvement of their viability and their vasculogenic potential. In addition, UAG improves CAC mobilization in diabetic patients. As validated in endothelial nitric oxide synthase (eNOS) knockout mice, UAG reverses the diabetic defect in CAC mobilization by restoring eNOS phosphorylation and MMP9 activity. Finally, specific UAG binding to CAC is shown. Thus, we present evidence that restoring the AG/UAG ratio protects CAC from diabetes-associated adverse metabolic effects. In addition, we provide the rational for clinical applications of UAG to improve vascular regeneration processes.

The synthetic derived of oleanoleic acid, CDDO-Im, displays antiangiogenic and angiopreventive properties through the blockage of the PI3k/Akt pathway

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Angiogenesis is a limiting step for the switch from a dormant to malignant state in tumor progression. The mutual interactions between tumourigenic cells and the surrounding tissue determine the formation of a milieu, the microenvironment, where tumours can grow and progress throughout the malignant process. Angiogenesis and inflammation are phenomena affecting the tumour-associated changes of the microenvironment, and can be considered common targets for many chemopreventive molecules. One of these agents is the triterpenoid CDDO that, together with its derivates, have anti tumor property against a variety of cultured tumour cell lines and in mouse xenograft models.
To further investigate the potential role of these compounds in cancer therapy we evaluated the effect of the Imidazole derivative (CDDO-Im) on angiogenesis in in vitro and in vivo settings. CDDO-Im displayed potent anti-angiogenic property even at nanomolar dosage in vivo similar to what shown for CDDO-Me, a molecule displaying a strong chemopreventive property, as previously showed by our group. Interestingly, although both agents derive from the same initial molecule, they affected two different molecular pathway on endothelial cells.
CDDO-Im seemed to act mostly on the PI3k/Akt pathway, while CDDO-Me affected the erk1/2 pathway. Taken together, these data support the findings that CDDO-Im and CDDO-Me exerted their pro-apoptotic effect via caspase 3 and via caspase 8, respectively..
In conclusion, we demonstrated a novel activity for CDDO-Im as inhibitor of tumor angiogenesis, and its effect on endothelial cells intracellular signalling. The different molecular targets of CDDO-Im and CDDO-Me can make them “interchangeable” agents in the clinical settings when the development of drug resistance occurs.

Polycystin-1 induces cell migration through PI3K and actin, while cell polarization through PKCζ and microtubules

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Autosomal Dominant Polycystic Kidney Disease is a common inherited disorder characterized by renal cyst formation. ~85% of cases are caused by mutations in PKD1, whose gene product, Polycystin-1 (PC1), is a large (520kDa) receptor involved in cell-cell/matrix interactions and in primary cilia functions.
We have previously shown PC1 is involved in migration process: human PC1 overexpression in renal epithelial cells (MDCKPKD1) induces actin cytoskeleton rearrangements and cell migration through PI3kinase activity (Boca et al, MBC 2007). Time-lapse wound-healing assay also uncovered that PC1 mediates linear oriented cell migration. To better study PC1 dependent polarized migration we performed immunofluorescences on wound-healing assay, considering polarized cells with Golgi in front of the nucleus in a 120° angle in the direction of the wound. In this way we demonstrated that PC1 induce polarization toward migration direction. This property is independent from PI3K activity and from actin cytoskeleton rearrangement, since PI3K inhibitors or actin depolimerizing drugs don't perturb cell polarization; it is rather dued to microtubular cytoskeleton regulation, since microtubule inhibitors perturbs cell polarization. We indeed noticed that PC1 induces microtubular elongation in a consistent direction with Golgi position, in a confluence-indepenent manner. Moreover we revealed a higher amount of acetylated tubulin (i.e. stabilized microtubules) concentrated around the Golgi toward the front of migration, in MDCKPKD1 compared to the control. With nocodazole-resistance assay we confirmed that stabilized microtubules are more in MDCKPKD1 and Pkd1+/+, compared to MDCK controls and Pkd1-/- fibroblasts, respectively.
In addition we identificated PC1 interaction with key regulator of cell polarity, PKCζ, whose inactive kinase dead form impairs cell polarization in MDCKPKD1. PC1 could also modulate the PAR polarity complex formation, since the amount of Par3 and Par6 proteins immunoprecipitated with PKCζ is different in Pkd1+/+ and Pkd1-/-.
Alltogether these data propose that PC1 regulates cellular factors and the microtubular cytoskeleton, necessary for Golgi positioning in cell migration.

HGF induces cell migration, ruffling and Rac activation through a novel signalling pathway linking DGKα to aPKCs and RhoGDI.

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Spatially-restricted activation of Rac small GTPase is crucial for directed cell migration. Diacylglycerol kinases (DGKs) convert diacylglycerol (DG) into phosphatidic acid (PA), thus acting as molecular switches between DG- and PA-mediated signalling. We previously showed that Src-dependent activation and plasma membrane recruitment of DGKα are required for growth factor-induced cell migration and ruffling, by promoting both activation and plasma membrane targeting of Rac.
Inhere, we unveil a still undescribed signalling pathway linking activation of HGF-receptor to Rac spatially-restricted activation at ruffling sites, involving DGKα-mediated plasma membrane recruitment and regulation of atypical PKCs, associated in a complex with RhoGDI and Rac.
The dynamics of Rac targeting to the plasma membrane are regulated by RhoGDI, which complexes with Rac and, upon its release, allows its localized activation. Indeed, we show that RacR66E mutant, which is unable to bind to RhoGDI, fails to be recruited to the plasma membrane upon HGF stimulation.
We show inhere that DGKα is required for HGF-induced RhoGDI plasma membrane recruitment and Rac release from the complex with RhoGDI, whereas the expression of constitutively-active myr-DGKα is sufficient to recruit both RhoGDI and Rac to ruffling sites in absence of growth factor stimulation.
The dissociation of Rac/RhoGDI complex has been recently reported to be induced upon RhoGDI Thr phosphorylation by PKCz, which is directly regulated by PA. We show inhere that PKCζ/ι associates with RhoGDI and that its activity is required for both HGF- and myr-DGKα-induced ruffle formation and Rac/RhoGDI recruitment to ruffling sites. In this context, we show that (i) DGKα inhibition prevents HGF-induced PKCζ/ι recruitment to the plasma membrane, (ii) myr-DGKα is sufficient to induce PKCζ/ι translocation, as well as specific cell treatment with PA, and (iii) DGKα inhibition does not prevent ruffle formation and RhoGDI recruitment to ruffling sites induced by constitutively-active myr-PKCζ.
Overall, our data indicate that HGF-induced activation of Dgkα, by generating PA in a spatially-restricted manner at nascent ruffles, is essential to direct the recruitment of PKCζ/ι, Rac and RhoGDI to the leading edge. There, PKCζ/ι-mediated regulation of RhoGDI function leads to Rac release and localized activation, thus promoting the extension of membrane ruffles, which constitute essential requirements for cell migration.

Impaired keratinocyte proliferative and clonogenic potential in transgenic mice overexpressing 14-3-3 sigma in the epidermis

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The balance between proliferation and differentiation is finely tuned in keratinocytes through the interaction of many different molecular mediators. Alterations in this equilibrium can lead to abnormal proliferation or differentiation. 14-3-3 sigma (sigma) is the epithelial-specific isoform of an highly conserved protein family involved in the control of different biochemical processes via the interaction with consensus sequences phosphorylated on protein targets. In the skin, sigma is highly expressed in differentiated keratinocytes and in vitro evidence indicates that its downregulation leads to keratinocyte immortalization. To define the role of sigma in epidermal homeostasis, we generated transgenic mice overexpressing this gene in basal proliferating keratinocytes.
Depending on sigma expression levels, transgenic mice manifest localized desquamating skin lesions associated with sparse hair loss and atrophic skin, or the early onset of wrinkles only. Moreover, both wound repair and hair re-growth are delayed in transgenic mice compared to controls. Microscopically, the epidermis of transgenic mice shows reduced thickness. BrdU analysis indicates that the number of proliferating keratinocytes is significantly reduced in the epidermis compared to wild-type littermates. Cultured keratinocytes from transgenic mice show reduced proliferation associated with increased features of a differentiated phenotype. Accordingly, immunohistochemical and western blot studies show a reduced amount of keratins associated with the proliferation (K14, K5, K15). Moreover, clonogenicity assessment indicates that transgenic mice have an impairment of keratinocyte stemness associated with increased senescence features. In search of putative pathways controlling cell proliferation, we found that transgenic keratinocytes are unresponsive to IGF-1 treatment and this effect correlates with lack of AKT phosphorylation. All together these findings indicate a role for sigma in the control of epidermal proliferation-differentiation switch and identify the IGF-1 signaling pathway as involved in such control. Other pathways potentially mediating sigma activity are under investigation.

Fos-Like 1 (FOSL1) regulates endothelial cell adhesion and migration

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Vasculogenesis and angiogenesis are essential for embryonic development, wound healing and cancer growth. These are complex processes resulting in a high-organized sequence of events, including cellular proliferation, migration and formation of primitive endothelial tubes. Fosl1 knockout mice die between E10.0 and E10.5, owing to vascular defects in extra-embryonic tissues. Performing the differentiation of endothelial cells with wild type or knockout embryonic stem cells we demonstrate that FOSL1 is not necessary for endothelial differentiation but it is required for the correct assembly of tube like structures in vitro. Primary human umbilical vein endothelial cells (HUVEC) silenced for FOSL1 by shRNA show a reduced ability to form vascular connective networks and to migrate. FOSL1 knockdown leads to a high-disorganization of the actin cytoskeleton and the focal adhesion proteins FAK and paxillin, however this disorganization is not due to alteration the cellular content of these proteins nor their phosphorylation states. Adhesion assays performed using a broad spectrum of cell-adhesive proteins demonstrates that FOSL1 silenced cells displays a drastically increased adhesion, only when plated on vitronectin, whereas the use of other substrates does not affect their adhesive proprieties. Expression analysis of the vitronectin receptors urokinase Plasminogen Activator Receptor (uPAR) and ?v?3 integrin show that these surface molecules are drastically altered both at the transcript and protein levels. Interestingly, we observed that FOSL1 silencing strongly downregulates the inducer of cell migration uPAR, whereas highly up-regulated the cell adhesion receptor ?v?3 integrin. These findings suggest that FOSL1 plays a crucial role in the fragile cellular equilibrium between the migrative and the adhesive phenotypes both necessary to orchestrate the angiogenetic process.

Blocking ErbB-3/p85 interaction: a new strategy to escape Herceptin resistance

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In carcinoma cells, alpha6beta4 integrin activates several key signaling pathways, and in particular PI3K/Akt, to sustain tumor survival. In part, the alpha6beta4 integrin exerts its effect regulating the expression of ErbB-3 receptor at the traslational level. ErbB-3 receptor possesses six binding sites for p85, the regulatory subunit of PI3K. The increment of its translation by alpha6beta4 integrin increases the formation of ErbB-2/ErbB-3 heterodimer promoting alpha6beta4-dependent activation of PI3-K/Akt pathway and the ability of this integrin to impede apoptosis. Further, the functional cooperation between beta4, ErbB-3 and P-Akt proteins plays an important role in sustaining the survival of ERbeta1-negative breast cancers during Tamoxifen therapy indicating that their concomitant expression is clinically relevant in predicting the response to Tamoxifen therapy in the adjuvant setting. Here we demonstrate that ErbB-3 is also involved in the mechanism of resistance to Herceptin treatment in beta4 positive breast carcinoma cell lines that overexpress ErbB-2 receptor. By the use of specific peptides able to physically impede ErbB-3/p85 interaction, we evaluated the responsiveness of the cells to Herceptin therapy. Treatment of MCF-7 and BT474 breast cancer cells with this peptide, which competes for the binding of ErbB-3 to the N-SH2 domain of p85, inhibits beta4-dependent PI3K and MAPK signalling pathways and enhances the responsiveness to Herceptin treatment. Interestingly, the combination of both treatments induces a significant apoptosis. These *in vitro* data show that the administration of a specific peptide against ErbB-3/p85 interaction in combination with Herceptin treatment could be a strategy to escape resistance in the *in vivo* therapy.

The binding of NCAM to FGFR1 leads to a specific cellular response mediated by receptor trafficking

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Neural Cell Adhesion Molecule (NCAM), which belongs to the Immunoglobulin family of Cell Adhesion Molecules (Ig-CAMs), is mainly expressed in the central nervous system, where it controls cell-cell adhesion and neurite outgrowth. It is also found in non-neural tissues, where its function is still unknown. Based on our previous observation that NCAM activates FGFR signaling in pancreatic beta tumor cells, we hypothesised that NCAM acts as a non-canonical ligand for FGFR. Indeed, we found that NCAM not only stimulates some of the known FGFR-mediated pathways, but it is also able to trigger FGFR-mediated cellular events that are remarkably distinct from those elicited by FGFs, the canonical ligands for FGFR. While FGF promotes cell proliferation via the classical Ras-Erk1/2 cascade, NCAM induces FGFR-dependent Src activation, resulting in cell migration. Moreover, NCAM, unlike FGF, induces the sustained activation of most FGFR effectors.
We also observed that the intracellular fate of FGFR differs between NCAM- and FGF- stimulated cells. Internalized FGFR is not ubiquitinated and degraded upon prolonged NCAM treatment, but is recycled back to the cell surface in a Rab11-dependent manner. Finally, FGFR-1 recycling is required for NCAM-induced sustained signaling and cell migration, pointing to the critical role of FGFR trafficking in dictating the cellular response evoked by receptor activation.
These findings provide the first demonstration that an immunoglobulin-like cell adhesion molecule can directly activate a receptor tyrosine kinase. Besides introducing a further level of complexity in the regulation of FGFR1 functions, our results suggest that the NCAM/FGFR interplay could be a potential player (and therapeutic target) in those diseases characterised by aberrant FGFR signaling, such as certain tumor types.

Met as a potential therapeutic target in basal-like breast cancer:
An in vivo approach in a stem-cell perspective

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Met overexpression has been associated to a highly invasive and poorly differentiated subtype of breast cancer, known as basal-like breast cancer. On the basis of gene expression profile it has been proposed that basal-like tumors derive from mammary stem/progenitor cells. The deregulation of pathways specific to mammary stem/progenitor cells may contribute to the generation of these tumors. In this perspective we investigated Met expression in normal mammary stem/progenitor cells and its role in proliferation, self-renewal and differentiation, and whether its overexpression leads to the formation of neoplastic lesions.
To address this issue we performed expression analysis on FACS-sorted mammary stem/progenitor cells and found that Met is highly expressed in the population of luminal progenitors (CD24highER-), whereas it is barely detectable in the basal cell compartment (CD24low) and in differentiated luminal cells (CD24high ER+).To assess the functional role of Met expression in the ER- progenitors, we evaluated their differentiation capabilities in 3D culture systems: when cultured in Matrigel, these cells underwent a differentiation process that led to the generation of polarized, single-cell layered alveolar-like structures with a hollow lumen. In the presence of exogenous HGF, the morphology of the colonies was dramatically modified: they have a stem-cell-like derived epithelial structure, and express a higher level of cytokeratin 14, marker of stem cell compartment, compared to the untreated cells. We explored the functional role of Met expression in mammary gland development by fat pad transplants experiments. In accordance with the in vitro findings, we observed that down-modulation of Met by shRNA in the transplanted cells interfered with the normal ratio of ER+/ER- luminal cells by increasing the population of ER+ differentiated cells with respect to the quota of ER- progenitors.
Expression analysis in tumors derived from a mouse model of basal-like breast cancer (BRCA1/p53 ko mice) revealed that Met is overexpressed in a subpopulation of CD24+ ER- cells with respect to the normal mammary epithelium. On the basis of these data we proposed that (1) Met activation is involved in the regulation of progenitors' fate and (2) may result in the block of luminal differentiation together with the re-acquisition of stem cell-like properties, underlying a biological role for Met in basal-like breast cancer and for Met inhibitors as a potential therapeutic approach.

Functional role of ArhGAP 15, a new RacGAP PI3K-dependent

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ArhGAP15 is a recently cloned RacGAP with a pleckstrin homology (PH) domain at C-terminal and a RhoGAP domain at N-terminal. The GAP domain of ArhGAP15 showed specificity towards Rac1 in vitro. The PH domain is required for ArhGAP15 to localize on plasma membrane. Coexpression analysis with bioinformatic tools revealed that ArhGAP15 coexists in , the unique class IB of PI3K that is specificallyγleukocytes with PI3K activated by G protein-coupled receptor and that has a central role in leukocyte migration. Here we show that ArhGAP15 appears localized in the cytoplasm in resting conditions and is translocated to the plasma membrane upon C5a stimulation in bone marrow-derived macrophages. Treatment with the PI3K inhibitor LY294002 significantly reduced ArhGAP15 membrane localization in stimulated cells, indicating that is membrane localization and activation is PI3K-dependent. These results indicate ArhGAP15 as a possible PI3K effector. To address the in vivo relevance of this protein, knock-out mice were generated. The loss of ArhGAP15 induces multiple alterations of leukocyte cellular behavior known to be under the control of Rac and PI3K.

Bimodal pattern of chemokine induced Rap1 activation: dissecting the molecular mechanisms

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The multi-step leukocyte extravasation process is governed by adhesion molecules and chemotactic factors dynamically interplaying in the presence of shear forces. Responsiveness to chemotactic ligands is mediated by G protein-coupled receptors (GPCRs) which are finely regulated by a well characterized family of cytosolic proteins, beta-arrestin 1 and 2. Recent evidence indicates that, in addition to playing a regulatory role in GPCR desensitization and internalization, beta-arrestins may contribute to GPCR signaling by functioning as scaffolds for the recruitment of signaling proteins into complexes with agonist-occupied receptors. On this basis, we investigated the physiological role of beta-arrestin 2 in chemokine-driven dynamics associated with leukocyte extravasation, with special interest to the activation of the Rap1 small GTPase, recently emerged as pivotal regulator of integrin function. The analysis of KC (Keratinocyte-derived Chemokine) the Rap1 activation profile in RBL (Rat Basophilic Leukemia) cells expressing mCXCR2 shows a bimodal kinetic, with the first peak at 30"/1' and the second at 5' after stimulation. RNA interference-mediated depletion of beta-arrestin 2 specifically inhibits the occurence of the second wave of Rap1 activation, whilst it has no effect on the early pick, thereby suggesting that the oscillations in the formation of Rap1-GTP are regulated by different molecular mechanisms. In order to elucidate the GEFs and GAPs involved in the GTPase activation we down regulated C3G (Rap1GEF) and Spa1 (RapGAP): the former doesn't seem to be involved as C3G depletion has no effect in KC-dependent Rap1-GTP formation, while Spa-1 has probably a role in the early activation peak. Since this oscillatory chemokine-induced Rap1 activation is present on other myeloid cell lines and fresh PMN's we are now translating our research to these more appropriated cells.

Identification and roles of cell substrates of intracellular mono-ADP- ribosylation

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Mono-ADP-ribosylation is a post-translational modification of proteins consisting in the transfer of the ADP-ribose moiety from NAD+ to a specific amino acid of an acceptor protein. The molecular aspects of ADP-ribosylation in living cells are not well defined and several of the endogenous substrates remain to be identified.
We aim at characterizing the physiological role of this reaction by delineating the enzymatic pathways (enzyme and substrates) linked to specific cellular processes. To this end, we have based the identification of ADP-ribosylated intracellular targets on a recently reported method involving a protein module (macro-domain) that recognizes ADP-ribosylated proteins (Dani et al., in press).
The 11 novel members of the poly-ADP-ribosyl-polymerase (PARP) family and some sirtuins are among the cellular enzymes for which ADP-ribosylation activities have been recently reported. Using ADP-ribosylation assays, we found that PARP12 is one of the more active enzymes and, therefore, we focused our attention on PARP12. To identify PARP12 substrates, we first performed ADP-ribosylation assays using extracts from HeLa cells transfected with PARP12 and then the modified substrates have been pulled-down using the macro-domain based method. Among the 25 putative substrates of PARP12, identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), we are validating some of the Rab family members, since they are also substrates of ADP-ribosylation catalysed by the exotoxin ExoS of Pseudomonas Aeruginosa (Barbieri et al., Traffic 2008).
Some of these Rabs are involved in the ER-to-Golgi transport and in the maintenance of the Golgi morphology. Interestingly, and connected to this, HeLa cells overexpressing PARP12 lost the classical Golgi ribbon organization, appearing instead as fragmented membranes. We are presently investigating the intriguing possibility that ADP-ribosylation (of Rabs) catalysed by PARP12 participates in the regulation of the Golgi complex structure.

Beta1 integrin controls EGFR signalling and tumorigenic properties of A549 lung cancer cells

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Increased levels of EGFR gene expression are observed in many types of cancer, including lung cancer, making this receptor a good therapeutic target. However recurrence and resistance to therapy require the development of novel treatment approaches. Our previous work demonstrates a tight co-operation between beta1 integrin and EGFR, required for signalling from the plasma membrane to the nucleus. Here, we silenced beta1 integrin in human non-small lung cancer A549 cells, that express high levels of EGFR and depend on EGFR activation for their growth. Cells silenced for the beta1 integrin are impaired in EGF-dependent activation of EGFR and downstream signalling such as ERK1/2 MAPKs and Akt, leading to a decreased in vitro proliferation. Moreover, beta1 integrin silencing increases sensitivity to cisplatin and gefitinib treatment. Interestingly, in in vivo experiments, silenced cells gave rise to a 70% reduction of tumour size in SCID mice. The resulting tumours showed defective EGFR signalling and a more differentiated phenotype. Taken together these results show that silencing the beta1 integrin affects EGFR activity, growth properties and responsiveness of lung cancer cells to pharmacological therapy, thus providing evidence for the relevance of the beta1 integrin/EGFR cross-talk in lung cancer.

A *Dlx5-Wnt5a* regulation involved in the control of GABAergic differentiation

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In the developing brain, migration and differentiation of neural progenitors is controlled by the cooperation of intrinsic (genetically determined) and extrinsic (environmentally determined) molecular regulations. How this interplay results in position- and time-specific differentiation of various neuronal types remains poorly known.
The Dlx homeogenes code for transcription factors related to distalless, are expressed in interneuron (IN) progenitors as well as differentiated GABA+ IN, and are essential for their migration and differentiation in the olfactory bulb (OB) and neocortex. In Dlx5-/- mice differentiation of IN is reduced, while migration is unaffected. We set forth to determine the molecular mechanism of Dlx5 control on this process, in vitro and in vivo.
We provide evidence that Wnt5a, a secreted signalling molecule of the Wnt family, is a target of Dlx5. In the absence of Dlx5, Wnt5a expression is reduced both in the OB and in cultured progenitors. Wnt5a transcription is activated by Dlx2 and Dlx5 via intronic homeodomain-response elements. Functionally, Wnt5a applied on slice cultures of the embryonic OB promotes IN differentiation. Wnt5a is known to signal independently of β-catenin and to activate alternative pathways, such as the JNK-dependent PCP pathway. Accordingly, JNK phosphorylation level is diminished in Dlx5 mutant OB, while β-catenin is unaffected.
In order to confirm the role of Dlx-Wnt regulation in GABA differentiation, we adopt a model of neural stem (NS) cells (PloS Biol 3: e283) that is able to maintain a Dlx hierarchical regulation and GABA+ differentiation potential similar to in vivo progenitors. In normal NS cells Wnt5a expression is upregulated following a neuronal differentiation signal, while in Dlx5 null cells this upregulation is delayed, accompanied with a reduced GABA+ and CR+ differentiation.
These data link the transcription functions of Dlx5 with the expression of the diffusible molecule Wnt5a in the olfactory brain, and establish the function of this pathway to promote interneuron differentiation. These data also account for a cell-non autonomous role of Dlx2 and Dlx5, stressing the notion that the local environment plays a fundamental role in determining the differentiated cell fate of neural progenitors.

Endothelial cell aging: altered expression of proteins involved in signaling of angiogenic activity

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Alterations of endothelium are associated with an increased risk in cardiovascular diseases; they are also involved in tumor angiogenesis, one of the main "rate limiting step" in tumor progression and spreading. For this reason in the last years several agents have been patented for the cancer therapy thanks to their ability to inhibit tumor angiogenesis and now angiogenesis is considered also a target prevention.
Since it has been demonstrated that cells that lose some phenotypic or functional features, due to the ageing process, are less prone to become cancerous cells, we evaluated the molecular changes occurring during aging in endothelial cells derived from Human Umbilical Vein (HUVECs) in early and late culture passages.
Interestingly, cells derived from male donors differed from those derived from females in terms of gene expression patterns. Several genes were regulated during the aging process, among which E-selectin and CCL2 showed a significant reduction of the expression rate during progressive passages. Angiopoietin 2 (ANG2) also seem to decrease during the endothelial cell senescence. In cells derived from female donors the observed expression rate was about 2-fold higher than in cells derived from male donors, suggesting a possible gender-specific difference in the aging process.
No gender-specific differences were observed in the expression of TGFßI, a gene induced by TGFß, although we observed an increased expression level in progressive in vitro passages.
Taken together, these data confirm that physiological endothelial cell senescence could be associated with a reduction in anti-angiogenic potential; this property suggests that some tumors could be less angiogenic in the elderly.

Mediating cAMP and PIP3 signaling by reciprocal regulation of PI3Kγ and PKA

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PI3Kγ is a phosphoinositide 3-kinase characterized by both lipid and protein kinase activity. It is activated by G-protein-coupled receptors and it negatively regulates heart contractility, by decreasing cAMP concentration activating a class III phosphodiesterase, PDE3B, through a kinase-independent mechanism. The PI3Kγ-dependent enhancement of PDE3B function was mediated by PKA which phosphorylates PDE3B and activates cAMP destruction. In this process, PI3Kγ plays a crucial scaffolding role by directly binding, through its N-terminal domain, the PKA RII subunit. PI3Kγ thus acts as a novel A-Kinase Anchoring Protein (AKAP), triggering a negative feedback loop that reduces PKA-mediated cardiac contractility. Within this complex, PKA and PI3Kγ are reciprocally regulated: PKA was found to phosphorylate PI3Kγ and limit its kinase activity upon β-adrenergic receptor (β-AR) stimulation. Consistently, β-AR-mediated Akt phosphorylation appeared to depend on PI3Kβ rather than PI3Kγ. Unexpectedly, cardiac PI3Kγ activity was down-regulated by PKA as an early response to mechanical stress but escaped this negative regulation in failing hearts. This suggests that cardiomyocytes tightly regulate PI3Kγ function in space and time and that dysregulation of such process correlates with heart failure.

Role of Diacylglycerol kinase alpha in Rab-coupling protein-driven integrin recycling and cell migration on 3D matrices

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A2780 ovarian tumor cells can be stimulated to move through 3D matrices either by the addition of osteopontin or by the expression of Rab25 and they do so by extending long invasive pseudopods in the direction of migration. We have recently shown that this type of migration requires a pathway that recycles integrins from endosomes to the plasma membrane. Moreover, this recycling pathway is dependent on the Rab11 effector Rab-coupling protein (RCP), which is recruited to the tips of invading pseudopods as the cells migrate. RCP contains a C2 domain, which has been shown to bind to phosphatidic acid (PA). This lipid second messenger can be produced in cells by two different enzyme families, diacylglycerol kinases (DGKs) and phospholipase Ds (PLDs), through the phosphorylation of diacylglycerol (DG) and the hydrolysis of phosphatidylcholine respectively. Here we show that Diacylglycerol kinase alpha (DGKα) is necessary for the transport of α5β1 integrin and Epidermal Growth Factor Receptor 1 (EGFR1) from endosomes to the plasma membrane, a step previously shown to be regulated by RCP (1). Conversely, DGKα knock-down did not affect the recycling of internalised transferrin, which is not an RCP-dependent event. We then proceeded to image the migration of A2780 cells as they move on a 3D fibrillar matrix, and found that DGKα was required for the extension of long invasive pseudopods and for the accumulation of RCP-containing vesicles at the tip of these structures when cells were induced to migrate by the addition of osteopontin. Although the expression of Rab25 induces a similar invasive phenotype to osteopontin, it was not affected by the downregulation of DGKα, but was completely dependent on PLD activity. Consistently, PLD was required for pseudopod elongation induced by Rab25 expression, but not by osteopontin treatment. Therefore, the production of PA, driven by different enzymes in different cellular contexts, is a crucial event in the signalling pathway regulating RCP function. Furthermore, the expression of a myristoylated and constitutive-active form of DGKα was sufficient to induce pseudopodial elongation and RCP recruitment. Finally, DGKα did not affect RCP association with α5 integrin and EGFR1, indicating that DGKα mediates a step downstream of RCP binding to α5, probably the delivery of the RCP-containing vesicles to the plasma membrane. Overall, these data demonstrate that DGKα, by producing PA in a spatially restricted manner, has a pivotal role in the regulation of the specific signalling pathway induced by osteopontin which leads to α5β1 integrin and EGFR1 recycling and eventually to tumour cell invasion through 3D microenvironments.

Integrin serve as signaling hub indipendently of ligand binding

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The urokinase-type plasminogen activator receptor (uPAR/CD87) is a non-integrin vitronectin cell adhesion receptor linked to the outer membrane leaflet by a glycosyl phophatidyl inositol (GPI)-anchor. The mechanism by which this receptor induces signaling and cell migration, lacking of direct contact with the cytoplasmic side of the plasma membrane, is a matter of debate and remains largely undocumented at the molecular level. We now present evidences that uPAR mediated vitronectin adhesion modulates cell signaling and migration by induction of a novel type of adhesion-induced, ligand-independent, "outside-in" signaling from the beta integrin subunits. Remarkably the combined disruption of the integrin binding site in vitronectin and of the matrix binding capacity of beta integrins fail to impair uPAR-induced migratory signaling. Nevertheless, the integrin still needs to be in an active conformation as evidenced by the blocking effect of allosteric inhibitory antibodies, and still has to contact cytoplasmic scaffolding proteins including talin and kindlin as documented by the deleterious effect of cytoplasmic tail mutations.
This novel signaling paradigm is fundamentally different from classical integrin "outside-in" signaling in the fact that the receptor providing the physical contact with the ECM (i.e. the anchoring receptor) is different from the integrin transducing the signal (i.e. the signaling receptor). In fact, signaling poses no identifiable requirements to the anchoring receptor in terms of structure, ligand type, or mode of membrane anchorage as long as it effectively connects the cell to the ECM. The apparent lack of specificity, strongly suggest that the signaling does not involve direct or "adaptor" mediated contacts between the anchoring and the signaling receptors and thus imply signaling by mechanotransduction.

Melusin is a mechanical stretch responsive protein interacting with Hsp90 and IQGAP1

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Melusin is a muscle-specific protein involved in triggering cardiomyocytes hypertrophy in response to pressure overload. Despite the absence of kinase domain, melusin is responsible for AKT and MAPKs activation in response to mechanical stretch. To define melusin mechanism of action, we searched for molecular partners involved in melusin dependent signal transduction. Here we show that melusin binds to Hsp90 and IQGAP1. Hsp90 is a multifunctional chaperone recently involved in regulation of several protein kinases, such as MAPKs and AKT. IQGAP1 has been described as a scaffold protein for the MAPK pathways. We disclose that melusin is part of a molecular complex which include Hsp90, IQGAP1, Raf1, MEK1/2 and ERK1/2. Moreover we found that the amount of melusin-associated MEK1/2 is regulated by mechanical stretch. Furthermore we showed that melusin possesses a chaperone activity per se. Based on these data we hypothesize that melusin, in association with Hsp90, controls mechanical stretch-induced heart hypertrophy by chaperoning MAPK signaling pathway.

A novel rab5-based signaling pathway participates in centrosome cohesion

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The centrosome is made of two connected centrioles that replicate at the G1/S transition. Duplicated centrosomes are held together until G2, when they separate and migrate to the opposite poles of the cell. Here, we report the identification of a Rab5-dependent pathway participating in centrosome separation at late G2/mitosis. We found that Rab5, a critical GTPase controlling membrane trafficking, is associated with the centrosome in human cells and that both the Rab5 GTPase activating protein, RN-tre, and the Rab5 GDP/GTP exchange factor, Rabex-5, localize at the centrosome. Reduction of the Rab5 activity, by simultaneous silencing of Rab5A, Rab5B and Rab5C, inhibited centrosome separation during G2 and reduced the distance between the spindle poles at mitosis. The molecular mechanisms downstream of Rab5 involve KIF3A, a kinesin motor protein that is also a component of the centrosome. We show that KIF3A binds to Rab5 and that it is required for proper centrosome separation. Consistently, KIF3A is recruited to centrosomes in a Rab5-dependent manner. We therefore propose that Rab5 has a function in the separation of duplicated centrosomes at G2/M through a novel effector mechanism, relying on the recruitment of KIF3A.

Ubiquitination threshold controls the EGFR signalling pathway

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Plasma membrane receptors can be endocytosed through different entry routes, including clathrin-dependent (CME) and -independent pathways (NCE). However, it is not clear why distinct pathways of internalization have evolved. One possibility is that they might differentially regulate receptors functions and fate. We recently found that this is the case for EGFR, that once is internalized via CME is by-and-large recycled to the cell surface, while when it enters into NCE it is committed to degradation. This has profound implications for signaling, as we demonstrate that CME, by skewing EGFR fate towards recycling rather than degradation, allows sustained signalling to take place. Thus CME of the EGFR is not an attenuator function, but rather a mechanism to prolong signaling [1]. Interestingly, data from our lab suggest that cells can react to different doses of EGF in strikingly different ways [2]. At low doses of ligand, the receptor (EGFR) is internalized almost exclusively through CME and it is not ubiquitinated; at higher concentrations of ligand a substantial fraction of the receptor is endocytosed through NCE, as the receptor becomes ubiquitinated. Thus, EGF concentration is critical for the final biological outcome. How can cells decode the information stored in stimulus strength? Preliminary results show that the interplay between phosphorylation and ubiquitination of the EGFR plays a major role in this process. Ubiquitination depends on phosphorylation, because three out of the nine EGFR need to be phosphorylated in order for the EGFR to be ubiquitinated. Interestingly, while phosphorylation increases linearly with EGF doses, ubiquitination does not start before the EGF concentration reaches a critical threshold. To gain insight into this phenomenon we are using a multidisciplinary approach, involving both molecular biology experiments and mathematical modeling. We have produced a series of cell lines expressing various mutant forms of EGFR, and a model that explains these data will be presented. [1] S. Sigismund et al., Dev Cell, 15, 209-19 (2008). [2] S. Sigismund et al., Proc Natl Acad Sci U S A 102, 2760 (2005).

ATM Kinase activity modulates sensitivity to death receptor induced apoptosis through the regulation of FLIP protein stability

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Ataxia Telangiectasia Mutated kinase (ATM) is a Ser/Thr kinase that plays a central role in the DNA damage response. Recently several reports point to ATM kinase as a player in several cellular responses, such as the oxidative stress response, the insulin pathway and the modulation of protein stability. We have recently identified a new role of ATM kinase in death receptor induced apoptosis. ATM kinase activity negatively modulates FLIP protein stability therefore enhancing death receptor sensitivity. Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of death receptor family, has been largely investigated as a potent tool to trigger apoptosis in cancer therapy because of its high specificity for cancer cells. One of the major targets for TRAIL resistance is the aberrant up-regulation of FLIP protein in some cancer cell lines, and indeed many approaches to downregulate FLIP have been proposed as valuable tool for combined therapy in several tumours. Here we present evidence that ATM activity is required for FLIP downregulation by DNA damaging agents and that genetic inhibition of ATM impairs TRAIL sensitization by DNA damaging agents such as 5-FU. These data point to ATM kinase activity as a crucial player in the molecular mechanism through which DNA damaging chemotherapeutic drugs and possibly IR increase TRAIL sensitivity in tumour cells. Moreover we also investigate the molecular mechanism through which ATM kinase activity may modulate FLIP protein levels. Here we present present data supporting the hypothesis that ATM kinase activity modulates FLIP protein levels through the proteasome-ubiquitin pathway. ATM kinase activation in response to DNA damage triggers FLIP protein ubiquitination and degradation.It has been recently reported that FLIP protein stability is modulated by the E3-ubiquitin ligase ITCH. Interestingly, in transient transfection experiments, we found that ATM kinase activity promotes ITCH-dependent FLIP degradation. Overall, these experiments suggest that ATM kinase activity modulates FLIP stability through the proteasome-ubiquitin pathway. Future experiments will help to clarify the molecular mechanism through which ATM kinase activity impinges on ITCH function.

Unacylated ghrelin recovers neovascularization in diabetes by targeting enos and redox signaling

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A relative excess of acylated ghrelin (AG), compared to the unacylated form (UAG), and an impaired circulating level of angiogenic cells (CAC) are common features in diabetes. In the present study we investigate the therapeutic potential of UAG in this setting. We demonstrate that UAG, by regulating the NADPH oxidase subunit Rac1, protects diabetic CAC from oxidative stress and from p53-mediated senescence like-growth arrest leading to an improvement of their viability and their vasculogenic potential. In addition, UAG improves CAC mobilization in diabetic patients. As validated in endothelial nitric oxide synthase (eNOS) knockout mice, UAG reverses the diabetic defect in CAC mobilization by restoring eNOS phosphorylation and MMP9 activity. Finally, specific UAG binding to CAC is shown. Thus, we present evidence that restoring the AG/UAG ratio protects CAC from diabetes-associated adverse metabolic effects. In addition, we provide the rational for clinical applications of UAG to improve vascular regeneration processes.

Defining the role of mTOR at the interface between cancer cell growth and immune surveillance

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The mammalian target of rapamycin (mTOR) regulates cell growth and proliferation integrating signals from nutrient and growth factor. In mammals, mTOR exists in two distinct protein complexes: mTORC1 and mTORC2, which reveal different sensitivity to the pharmacological inhibitor rapamycin. While initially identified as antifungal and immunosuppressant, rapamycin later proved potent cytostatic effects able to arrest transformed cells in the G1 phase of the cell cycle. Hence rapamycin derivatives are currently under evaluation in anti-cancer therapy. This is in spite their potential immunomodulatory effect. We report that pharmacological inhibition of the mTORC1 pathway efficiently prevents phosphorylation of its downstream targets (p70 Ribosomal S6 kinase and 4EBP-1), but only transiently arrest cell cycle progression. Indeed, by tracing single cell proliferation over time we found that in the presence of nutrients both primary and transformed cells overcome mTORC1 inhibition and divide through mTORC1 independent, MAPK and p90Ribosomal S6 kinase (RSK) dependent signaling pathways. In addition to mTORC1, TCR and CD28 engagement on the surface of transformed and primary T lymphocytes also recruits mTORC2, leading to AKT/PKB phosphorylation (Ser 473). While poorly effective against cell proliferation, blockade of mTORC-dependent signaling hindered T cell receptor (TCR) and CD28-driven primary T cells polarization. This reflected the ability of the drug to prevent early transcription of IFN-γ and IL-4 gene, which was best explained by the ability of mTOR to delay cell cycle entry, translation of T-bet, a transcription factor critical for timely IFN-gamma gene transcription, and upregulation of STAT-3 and STAT-4, also important in T cell differentiation. We are currently investigating the possibility that mTOR controls epigenetic events taking place soon after activation and concurrent with cell cycle transition, determinant for lymphocyte polarization.

Preclinical validation of predictive biomarkers for Met therapeutic inhibition

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The tyrosine kinase receptor Met drives the biological process known as invasive growth, is mutated or amplified in certain types of cancer and is the target of small molecules inhibitors currently tested in clinical trials. Yet, there is no systematic knowledge of which effectors are potentially involved in determining therapeutic response to Met inhibitors. In a medium-scale phosphoproteomic approach, Met inactivation with the specific inhibitor PHA-665752 in the Met-addicted GTL16 cell line modulates a variety of phosphoproteins that uniquely include Ras-dependent transducers, such as those in the RAF-MEK-ERK cascade and the PI3K-AKT axis (the "biochemical response signature"). On the basis of this knowledge and in order to further characterize the functional significance of the transducers likely involved in the response to Met inhibitors we designed two additional approaches. We investigate the response of GTL16 cells to treatment with PHA with an unbiased microarray strategy followed by ad hoc computational tools (i.e. GO annotations and Gene Set Enrichment Analysis, GSEA): in particular, consistently with our phosphoproteomic profile, GSEA analysis retrieved gene sets related to upstream inhibition of Ras-dependent signals including signatures derived from K-RAS knock-down. Moreover the functional importance of Ras-dependent transducers has been validated in inhibition and overexpression experiments: (a) using PI3K/AKT- and MEK-specific inhibitors either individually or in tandem, we showed a depletion in the phosphorylation levels of the respective substrates p70S6K and ERK1/2 and a reduction in the accumulation of GTL16 cells in growth assays, indicating that inhibition of these downstream pathways is sufficient to recapitulate the effect of upstream Met inhibition; (b) conversely, overexpression of constitutively active forms of B-RAF (B-RAFV600E) and/or AKT (AKTMyr) in a vast panel of Met-addicted cell lines was able to rescue PHA-induced growth inhibition, both in biochemical and biological assays. Thus, the whole set of data collected i) provides a nonredundant signature of Ras-dependent signals that appears to be necessary and sufficient to drive responsiveness to Met inhibition. Moreover, it offers ii) some insight into the mechanisms likely responsible to confer resistance to targeted agents and iii) a starting point for the identification of novel biomarkers to be used in the clinics to portrait ideal candidates for treatment.

Identification of the cellular targets and mechanisms of action of the glycerophosphoinositols

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The glycerophosphoinositols (GPIs) are water-soluble metabolites produced by a phospholipase A_2 (PLA_2) activity through two sequential deacylation reactions from the membrane phosphoinositides [1]. Among these GPIs, glycerophosphoinositol 4-phosphate promotes actin cytoskeleton reorganisation through the activation of the monomeric GTPases Rac1 and RhoA [2,3]. Dynamic changes in the actin cytoskeleton are central to a wide variety of cellular events, including cell motility, adhesion and phagocytosis, and tumour-cell invasion. With the aim of defining the mechanisms of action of the GPIs, we have used newly synthesized glycerophosphoinositol (GroPIns) derivatives with a biotin substituent bound on different positions of the glycerol backbone (biotinylated-GroPIns). The localisation of these biotinylated-GroPIns in different cell lines was investigated. Using confocal immunofluorescence, we have seen that biotinylated-GroPIns staining is diffuse within the cytosol, with specific localisation in the nucleus also seen. We are also using a proteomic approach to identify and characterise the cellular targets involved in the functions of the GPIs. The use of affinity chromatography with mouse-brain cytosol and a Sepharose-based matrix with covalently bound GroPIns has led to the identification of several interactors. A different approach based on the biotinylated-GPIs and streptavidin-conjugated affinity resins has also been developed. Altogether, we have identified 74 putative interactors, which we are selecting and validating. These can be divided into clusters of proteins involved in cell signalling, cytoskeleton organisation, protein folding and enzymes of metabolic processes. We are presently focussing on the components of signalling cascades that might be directly activated by the GPIs.

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A macromolecular signaling complex formed by the beta1 integrin and hERG1 channels is assembled in colorectal cancer cells: molecular definition and functional role

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Increasing evidence indicates that the expression and activity of different ion channels mark and regulate different aspects of cancer progression. As is being increasingly recognized, some of these roles can be attributed to signaling mechanisms, often independent of ion flow. Work in our lab has established that K+ channels encoded by the human ether-a-gò-gò-related gene 1, hERG1 channels, are expressed in several types of human cancers (including coloretal cancers), where they exert pleiotropic effects, triggering and modulating intracellular signaling cascades. This role depends on the formation, on the plasma membrane of cancer cells, of macromolecular complexes with integrin receptors. It is still debated whether the hERG1 protein inside the complex functions since it allows ion flux, or through a conformational coupling with the partner protein(s). We report here that hERG1 channels form a molecular complex with beta1 in colorectal cancer cells in the presence of serum or after adhesion onto Fibronectin. The complex did not occur in suspended cells but was rapidly triggered by the addition of a beta1 stimulating antibody (TS2/16). Once formed, the beta 1/hERG1 complex triggered intracellular signaling, finally regulating the transcriptional activity of the Hypoxia-inducible factor 1 (HIF-1) and hence the VEGF secretion by colorectal cancer cells. To test the conductive vs non conductive role of the channel protein inside the complex, we studied a non conductive hERG1 mutant (G628S mutation, gently provided by Dr. J. Mitcheson, University of Leicester, UK). The non conductive hERG1 mutant was stably transfected into HEK293 and colorectal cancer cells. Due to the dominant negative effect of the G628S mutant on wild type hERG1 currents, G628S colorectal cancer cells do not produce any detectable hERG1 currents. Experiments aimed at evaluating the proliferative and invasive characteristics of cells expressing wild type or G628S hERG1 channels, are ongoing.