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Dissecting signaling and functions of adhesion G protein–coupled receptors

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G protein–coupled receptors (GPCRs) comprise an expanded superfamily of receptors in the human genome. Adhesion class G protein–coupled receptors (adhesion-GPCRs) form the second largest class of GPCRs. Despite the abundance, size, molecular structure, and functions in facilitating cell and matrix contacts in a variety of organ systems, adhesion-GPCRs are by far the most poorly understood GPCR class. Adhesion-GPCRs possess a unique molecular structure, with extended N-termini containing various adhesion domains. In addition, many adhesion-GPCRs are autoproteolytically cleaved into an N-terminal fragment (NTF, NT, α-subunit) and C-terminal fragment (CTF, CT, β-subunit) at a conserved GPCR autoproteolysis–inducing (GAIN) domain that contains a GPCR proteolysis site (GPS). These two features distinguish adhesion-GPCRs from other GPCR classes. Though active research on adhesion-GPCRs in diverse areas, such as immunity, neuroscience, and development and tumor biology has been intensified in the recent years, the general biological and pharmacological properties of adhesion-GPCRs are not well known, and they have not yet been used for biomedical purposes. The "6th International Adhesion-GPCR Workshop," held at the Institute of Physiology of the University of Würzburg on September 6–8, 2012, assembled a majority of the investigators currently actively pursuing research on adhesion-GPCRs, including scientists from laboratories in Europe, the United States, and Asia. The meeting featured the nascent mechanistic understanding of the molecular events driving the signal transduction of adhesion-GPCRs, novel models to evaluate their functions, and evidence for their involvement in human disease.

Keywords: G protein–coupled receptors; GPS motif; autoproteolysis; molecular and genetic analysis

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Introduction

The biennial adhesion-GPCR workshops evolved from a European grassroots initiative that began in 2002 and have been fostering informal communication on topics concerning adhesion-GPCR research. The workshops slowly evolved from gatherings of a group of immunologists working on the founding adhesion-GPCR class members, F4/80 and CD97, to the only international meeting solely dedicated to adhesion-GPCR research. This year the tenth anniversary workshop gave more than 20 laboratories a platform on which to exchange their latest insights into the biology of this poorly understood receptor class. At the same occasion, the Adhesion-GPCR Consortium (AGC) was formed by the constituting member assembly (http://www.adhesiongpcr.org). The AGC will represent and disseminate adhesion-GPCR research and coordinate concerted funding initiatives on the topic. The workshop program was divided into (1) evolutionary aspects of adhesion-GPCRs, (2) signaling of adhesion-GPCRs, (3) adhesion-GPCRs in development, (4) adhesion-GPCRs in neurobiology, and (5) adhesion-GPCRs in disease.

The origin of the adhesion-GPCR family

Helgi Schiöth (Uppsala University) introduced his studies on the origin of the adhesion-GPCR class of seven-span transmembrane (7TM) receptors. The adhesion G protein–coupled receptors (GPCRs) are the second largest family of GPCRs, with genes containing multiple exons that are presumed to have arisen through several mechanisms.[1,2] Adhesion-GPCRs are of ancient origin and are found in several eukaryotes that include most of the vertebrates, the closest relatives to the vertebrates (*Ciona intestinalis* and *Branchiostoma floridae*), and the most primitive animals (*Nematostella vectensis* and *Trichoplax adhaerens*; Fig. 1).[3] Intriguingly, gene mining in amphioxus *B. floridae* has revealed several novel adhesion-GPCR domains such as somatomedin B, kringle, lectin C-type, SRCR, LDLa, immunoglobulin I-set, CUB, and TNFR, typically not found in the mammalian receptors.[4] Further, unique adhesion-GPCRs have been identified in urochordates (*C. intestinalis*) and in the *Strongylocentrotus purpuratus* (sea urchin) genome, and some of these are species specific. There are at least 21 adhesion-GPCRs in *C. intestinalis* that possess just the GPCR proteolysis site (GPS) proteolytic domain in the N-termini, while in the sea urchin there are 40 adhesion-GPCRs containing multiple leucine-rich repeats (LRRs) but lacking GPS sites.[5,6] Furthermore, comprehensive analysis of the entire set of adhesion and related secretin, and Methuselah groups of GPCRs provided the first evolutionary hierarchy among the five main classes of vertebrate GPCRs. Schiöth's group provided convincing evidence that the secretin GPCRs descended from the family of adhesion-GPCRs, probably from group V of the adhesion-GPCRs.[7] Moreover, they clarified the origin of the adhesion-GPCRs by providing the first evidence for the presence of adhesion-GPCR homologues in fungi.[8] This study estimated that the adhesion-GPCRs evolved from *Dictyostelium* cAMP receptors before the split of unikonts from a common ancestor of all major eukaryotic lineages.[8] In addition, they mined the close unicellular relatives of the metazoan lineage *Salpingoeca rosetta* and *Capsaspora owczarzaki*. These species have a rich group of the adhesion-GPCRs that provided additional insight into the first emergence of the N-terminal domains of the adhesion family.[8] Prime examples are the emergence of the characteristic adhesion-family domains, GPS and the Calx-β domain in *C. owczarzaki*, and the EGF-CA domain in *S. rosetta*.[8] Further, Schiöth analyzed the hemichordate *Saccoglossus kowalevskii* (acorn worm), which serves as an important model organism for developmental biologists to understand the evolution of the central nervous system (CNS). Unlike vertebrates that have a centralized nervous system, the acorn worm has a diffuse nerve net. Despite this, the acorn worm contains well-conserved orthologues for several of the adhesion family members with a similar N-terminal domain architecture. This is particularly apparent for those genes responsible for CNS development and regulation in vertebrates (Krishnan, A., *et al.* unpublished data). Overall, adhesion-GPCRs have a remarkably long and complex evolutionary history that can be traced down to the common ancestors of metazoa and fungi (Fig. 1). Knowledge of the origin and evolution of the unique N-terminal domain architectures of these genes may offer opportunities to better understand their functional roles and to aid deorphanization.

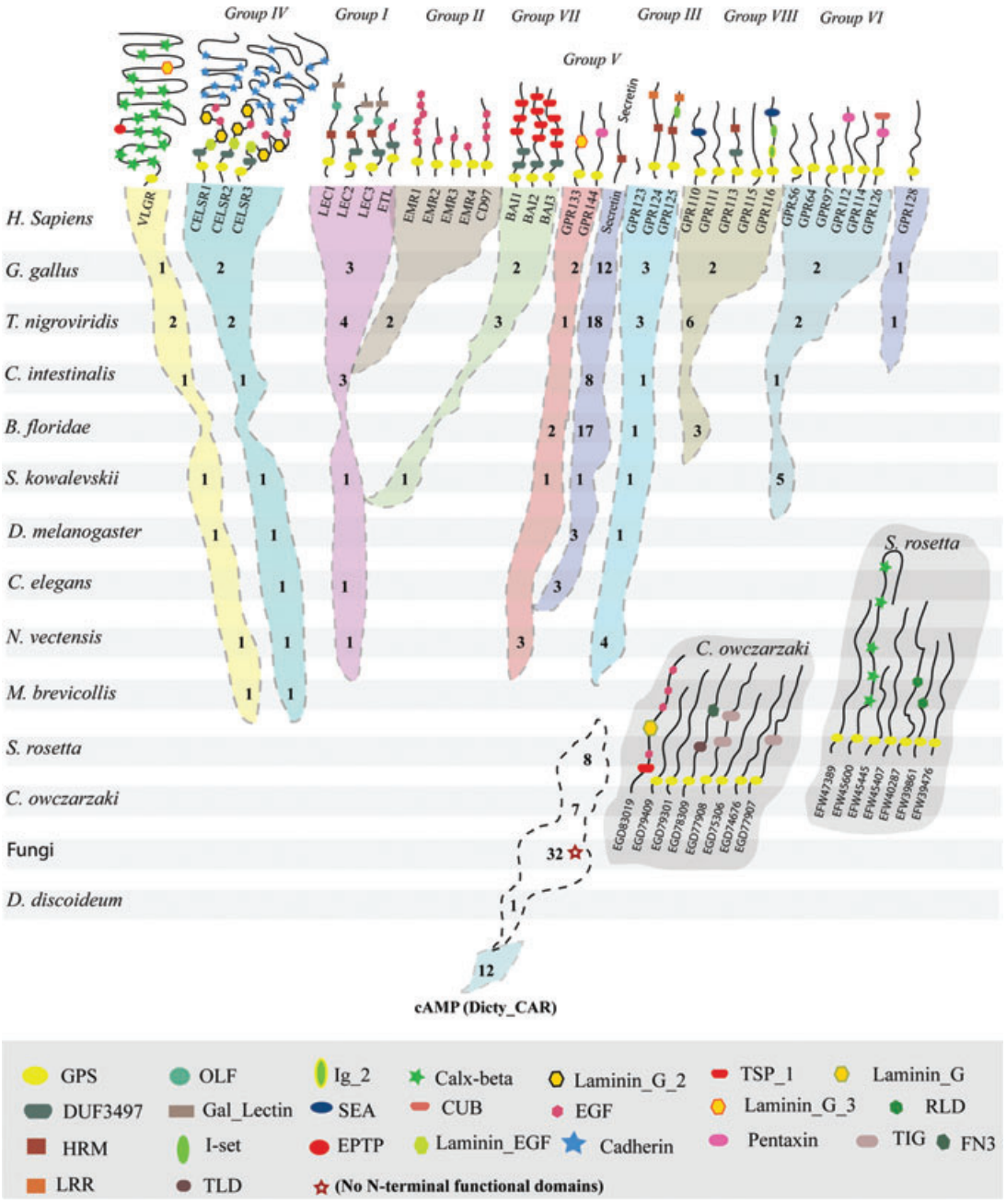

Figure 1. Schematic representation of the evolutionary history of adhesion-GPCRs. The figure summarizes the number of adhesion-GPCR family sequences found in each group across species. Each row denotes a species, and the colored fields are the adhesion-GPCR groups. N-terminal domains are shown for the human adhesion-GPCR genes. The N-terminal domain architecture of the unclassified novel adhesion-GPCRs found in *C. owczarzaki* and *S. rosetta* is shown separately. The red-colored star represents the absence of N-terminal domains for the novel fungi homologues.

Signaling of adhesion-GPCRs

Intense focus during the 6th Adhesion-GPCR Workshop was directed toward the mechanistic understanding of adhesion-GPCR signal transduction in relation to the structural components of adhesion-GPCRs. A group of researchers presented their findings on details of the molecular signaling mechanism of different adhesion-GPCRs, providing a comprehensive overview of methods, models, and receptors currently used to understand adhesion-GPCR signal transduction.

The GPS motif: 15 years of studies

Alexander Petrenko (the Shemyakin-Ovchinnikov Institute of the Russian Academy of Sciences), summarized previous research into the structural hallmark of adhesion-GPCRs and the GPS, which lies at the heart of connecting receptor structure with function. Purification and molecular cloning of the calcium-independent receptor of latrotoxin (CIRL/latrophilin/CL) revealed the unusual composition of two heterologous subunits that derive from the endogenous cleavage of the precursor protein at the extracellularly oriented site close to the first transmembrane segment of the CIRL heptahelical core. A cysteine-box motif surrounding this site of cleavage appeared to be conserved in the adhesion-GPCR family and in a few adhesion-like single-span transmembrane proteins. Similarly to the CIRL, all analyzed proteins with this motif appeared to be proteolyzed. Moreover, when mutations were introduced in this region, receptors were no longer cleaved. They therefore named this motif the *GPCR proteolysis site*, or GPS.[9] In addition to four cysteine residues, the GPS motif also contains two conserved tryptophans and strong preferences in other residues within the motif. GPS-defined cleavage of the CIRL and other adhesion-GPCRs takes place in the endoplasmic reticulum, thus avoiding furin-mediated proteolysis. Two major issues were addressed in further studies of the GPS in the laboratories of Petrenko and others. The first issue was the mechanism of the proteolysis; the second was the structure of the resulting protein products. For the CIRL, Petrenko and colleagues showed that the presence of the GPS motif was necessary for cleavage. With truncated mutants, they demonstrated the cleavage of a large CIRL fragment containing the GPS and a neighboring latrotoxin-binding domain, which is weakly homologous to the corresponding region in adhesion-GPCR BAI. However, they failed to observe cleavage of the recombinant protein with the GPS motif only. No protease involved in the GPS cleavage has been identified. On the basis of the presence of the *cis*-proteolysis signature, it was proposed that the cleavage is autocatalytic.[10] Yet, in an *in vitro* system they found that GPS cleavage can be regulated indicating a role for either a protease or some chemical cofactor. In their original CIRL description, they showed that the two cleavage products, p120 and p85, are tightly bound. However, later, p120 and p85 were found to overlap but only partially, thus suggesting their independent localization on the membrane.[11] They observed a separate soluble p120 fragment, but it was due to a secondary extracellular cleavage of the complex. Interestingly, the two-subunit complex can be dissociated under harsh conditions *in vitro*, but the fragments will not reassociate. Also, an extracellular peptide fragment of p120 that binds to p120 in native CIRL complexes would not bind to recombinant p120. All these observations received explanations in a recently published study on the structure of cleaved and uncleaved GPS-containing protein fragments of CIRL and BAI.[12] The GPS motif is part of a larger fold that was named the *GAIN domain*. Within this domain, the fragment with the GPS motif is tightly embedded into a larger structure, and there are no major structural changes or dissociation upon the cleavage. Future studies will have to address further the functional significance of GPS proteolysis and clarify its mechanism.

A novel and evolutionarily conserved domain of adhesion-GPCRs mediates autoproteolysis

Demet Araç (Stanford University) introduced her work on the structural elucidation of the GAIN domain. Unlike other GPCRs, adhesion-GPCRs have large extracellular regions that are autoproteolytically cleaved from their seven-pass transmembrane regions at a conserved GPS.[9] Previously, it was believed that the so-called stalk region that precedes the GPS motif of all adhesion-GPCRs was nonfunctional and unstructured. Unexpectedly, Araç discovered that the GPS motif itself does not constitute an autonomously folded domain, but rather forms a single folded domain together with the so-called stalk region. Thus, the ~40 residue GPS motif is an integral part of a much larger ~320 residue domain that they termed the *GPCR-Autoproteolysis INducing*

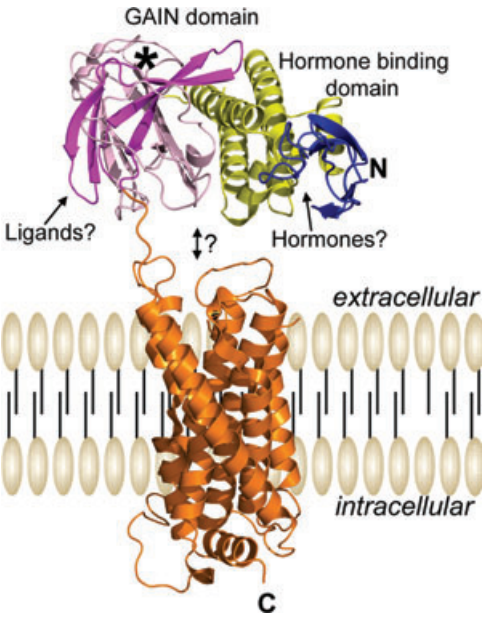

Figure 2. All adhesion-GPCRs have an extracellular GAIN domain that precedes the first transmembrane helix. The GAIN subdomain A is colored yellow. The GAIN subdomain B is colored light pink. The GPS motif, which is part of subdomain B, is colored magenta. The HormR domain (which exists in 12 of human adhesion-GPCRs) is colored blue. The modeled transmembrane helices are colored orange. The cleavage site is indicated with an asterisk. Possible interactions are indicated with a question mark.

(GAIN) *domain*. Araç and colleagues determined the crystal structures of GAIN domains from two distantly related adhesion-GPCRs, CL1 and BAI3, and revealed a conserved novel fold that was previously unidentified (Fig. 2).[12]

Strikingly, the GAIN domain is the only extracellular domain shared by all 33 human adhesion-GPCRs and all five human polycystic kidney disease proteins. Database searches have revealed that primitive organisms, such as *Dictyostelium discoideum* that arose early in evolution before animals emerged, encode GAIN domains, although they lack most other autoproteolytic domains, important adhesion and signaling domains, and critical signaling pathways. These findings show that the GAIN domain is a widespread and conserved autoproteolytic domain in higher eukaryotes as well as in ancient organisms.

Functionally, the entire GAIN domain is both necessary and sufficient for autoproteolysis, as determined by deletion experiments of CL1. Araç *et al.* performed extensive mutagenesis of the CL1 cleavage site and revealed the unique structural features of the GAIN domain that enable self-cleavage. Autoproteolysis occurs between the last two β-strands of the GAIN domain in a short and kinked loop, suggesting an autoproteolytic mechanism whereby the overall GAIN domain fine-tunes the chemical environment in the GPS to catalyze peptide bond hydrolysis. The GAIN domain is the locus of multiple human disease mutations, including cancer, autosomal dominant polycystic kidney disease, and bilateral frontoparietal polymicrogyria. The disease-causing mutations on the GAIN domains may interfere with autoproteolysis function or other functions of the GAIN domain such as ligand binding.

Two properties of the GAIN domain–mediated autoproteolysis make it unique and intriguing. First, all GAIN domains always immediately precede the N-terminal transmembrane helix by a short linker and are in close association with the signaling transmembrane domains (Fig. 2). Second, in contrast to most other autoproteolytic domains, upon autoproteolysis, the GAIN domain remains attached to the membrane-embedded regions of the protein. These observations naturally lead to the hypothesis that the GAIN domain may regulate receptor signaling via the transmembrane helices; and GAIN domain–mediated autoproteolysis has a complex mechanism of action. Indeed, deletion experiments, reported by others, indicate that the GAIN domain may have an inhibitory role on GPCR signaling.[13]

The hormone receptor (HormR) domain is the second most frequently observed domain in adhesion-GPCRs (found in 12 of 33 human adhesion-GPCRs). However, no hormones have yet been identified to bind adhesion-GPCRs. Remarkably, Araç's HormR domain crystal structures from CL1 and BAI3 revealed an unusually high structural similarity (0.7 Å r.m.s.d.) to the genuine hormone-binding domain of the corticotrophin-releasing factor receptor (CRFR), suggesting that adhesion-GPCRs may be hormone receptors.

In summary, Araç's work has redefined the poorly understood adhesion-GPCR class, showing that members of this family share a large, unique, and widespread autoproteolytic domain that may be involved in downstream signaling and in human disease.

Activation of adhesion-GPCRs by an endogenous tethered ligand

Simone Prömel (University of Leipzig) introduced her studies on the GPS motif of latrophilins, which have shed light on the molecular interactions that the GAIN/GPS element of adhesion-GPCRs participates in. Latrophilins (LPHN/CL/CIRL) have been characterized to be one of the receptors for α-latrotoxin, a component of the black widow spider toxin.[14] Binding of α-latrotoxin to LPHN1 leads to calcium-independent release of neurotransmitters in neurons.[15] Besides CELSR/Stan/Flamingo, latrophilins are the only members of the adhesion-GPCR family that are present in vertebrates and invertebrates. They can be found throughout several phyla and species, making them prototypic for the adhesion-GPCR class and suggesting that they have essential roles that are conserved in all bilaterians. Three latrophilin homologs exist in the mammalian genome (*lphn1–3*), whereas the nematode *Caenorhabditis elegans* contains two homologs, *lat-1* and *lat-2*. A *lat-1* null mutant causes variable morphogenic defects during embryonic and larval development, leading to embryonic and larval lethality. Previous studies indicate that LAT-1 participates in the control tissue polarity during embryogenesis.[16]

The molecular mechanisms of LAT-1 signaling remain elusive. Very few *in vivo* approaches to address such questions have been developed thus far. However, Prömel and colleagues employed an *in vivo* assay in *C. elegans* to assess molecular function of the latrophilin homolog LAT-1. The assay is based on transgenic rescue of *lat-1* mutant phenotypes by a wild-type or a modified *lat-1* copy, which allows analysis of structure–function relationships in the biological context of the receptor without detailed knowledge of input or output signals.

Using this assay, Prömel performed a comprehensive receptor analysis indicating that LAT-1 signals via two different types of interactions.[17] The first mode requires the presence and structural preservation of the GPS—which is an integral part of the GAIN domain[12]—and 7TM domains. By contrast, the other interaction is independent from the 7TM/C terminus of the receptor. Importantly, for both modes of receptor activity the GPS is essential. On a more mechanistic level, Prömel and colleagues uncovered that the GAIN/GPS domain mediates receptor activity by interacting with the 7TM of the receptor, consistent with its function as an endogenous tethered ligand of the 7TM domain (Fig. 3). This finding suggests that the GAIN/GPS structure might exert a similar function among several, if not all, adhesion-GPCRs by modulating the signaling of the 7TM domain.[17] Further, the assay system allowed intermolecular complementation experiments with pairs of LAT-1 receptor variants *in vivo*. These experiments revealed that the GPS cross-interacts with the 7TM domain of a homologous partner receptor, likely in a dimeric complex, for the 7TM-dependent function (Fig. 3). Prömel also tested the requirement of GPS proteolysis, which cleaves latrophilins and most other adhesion-GPCRs autoproteolytically.[10] Thus far this cleavage event has been assumed to be essential for receptor function.[18] In contrast to that assumption, Prömel and colleagues showed that GPS cleavage is not essential for receptor function.[17] Finally, the GAIN/GPS structure is required for a second function in *C. elegans* fertility that operates independently of the 7TM domain, indicating the molecular versatility of the GAIN/GPS region in adhesion-GPCRs (Fig. 3).[17]

The work by Prömel describes novel insights into adhesion-GPCR function on a molecular level and analyses under *in vivo* conditions. These insights, based on a complete receptor analysis, are the first steps toward a better understanding of the entire class of adhesion-GPCRs and a general mechanism for their mode of signaling. In future studies both modes of receptor activity, 7TM-dependent and -independent, and their impact on latrophilin function, will be analyzed in more detail. Additionally, current research by Prömel addresses the question of how GPS and the seven-transmembrane domain might interact to mediate function and which other interaction partners play a role.

G protein–mediated signal transduction of adhesion-GPCRs

Ines Liebscher (University of Leipzig) demonstrated a high-throughput approach to determine G protein–mediated signal transduction of adhesion-GPCRs. Over the last several years attempts have been made to unravel the issue of signal transduction of adhesion-GPCRs. Although several ligands as interacting partners with these receptors had been identified, activation of specific intracellular signal cascades remained obscure. There were

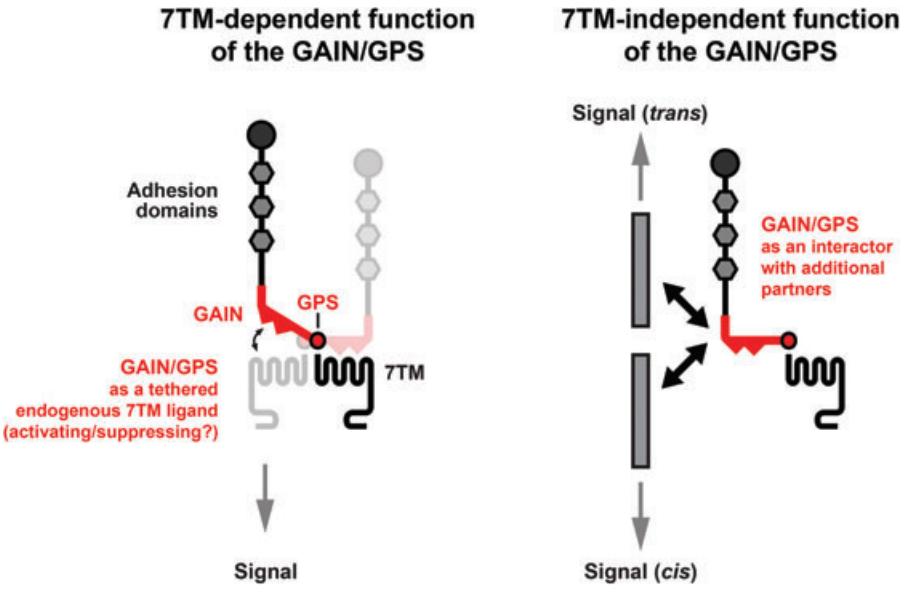

Figure 3. The GAIN/GPS domain of the nematode adhesion-GPCR LAT-1 serves two different signals. Left panel: in the 7TM-dependent mode, the GAIN/GPS domain functions as a tethered endogenous ligand of the 7TM domain. This interaction could occur in a dimeric complex of two homonymous receptor molecules, where 7TM the domain is cross-activated by the GAIN/GPS of the partner molecule, a mechanism akin to the activation of receptor tyrosine kinases. Right panel: in the 7TM-independent mode, the GAIN/GPS interacts with additional partners to transduce a separate signal (forward or reverse).

only a few reports on intracellular signaling mechanisms of adhesion-GPCRs. It was shown that latrophilin 1, the prototypic adhesion-GPCR, induced intracellular Ca^{2+} signaling upon interaction with the exogenous ligand α-latrotoxin.[19] GPR56 appeared to activate the $G\alpha_{12/13}$ protein/Rho-pathway after stimulation with an antibody against the ectodomain.[20] BAI1 recognized phosphatidylserine and could directly recruit a Rac-GEF complex to mediate the uptake of apoptotic cells.[21] However, clear evidence of intracellular signaling for most adhesion-GPCR via G proteins is still missing.

Bohnekamp and Schöneberg have recently shown that overexpression of the adhesion-GPCR GPR133, which is associated with adult height and the RR interval duration in an electrocardiogram, activates $G\alpha_s$, leading to an increase of cAMP levels.[22] $G\alpha_s$ protein–coupling of the basally active GPR133 was verified by $G\alpha_s$ knockdown with siRNA, overexpression of $G\alpha_s$, co-expression of a chimeric $G\alpha_{qs4}$ protein that routes receptor activity to the phospholipase C/inositol phosphate pathway, and by missense mutation within the transmembrane domain. Liebscher's data provided strong evidence to suggest that this member of the adhesion-GPCR family functionally interacts with the $G\alpha_s$/adenylyl signaling cascade. Further analysis showed that the presence of the N terminus and the cleavage at the GPS are not required for G protein signaling of GPR133. Liebscher has extended these investigations to other family members in order to study both the generality and specificity of G protein–mediated signal transduction of adhesion-GPCRs. Preliminary data indicate that GPR116, GPR123, GPR124, and GPR126 also couple to the $G\alpha_s$/adenylyl cyclase pathway. GPR115, GPR116, and GPR126 appear to activate $G\alpha_i$, and GPR115 is the only adhesion-GPCR so far that couples to the $G\alpha_q$/phospholipase C pathway. Recently, $G\alpha_s$ protein coupling was verified for GPR114 and GPR133, whereas GPR97 showed $G\alpha_o$ coupling.[23] These new data prove that classical receptor/G protein interaction is a common feature of adhesion-GPCR signaling.

A tethered inverse agonist model for activation of adhesion-GPCRs expressed on enteroendocrine cells

Thue Schwartz (University of Copenhagen and the Novo Nordisk Foundation Center for Basic Metabolic Research) described the impact of adhesion-GPCR signaling on enteroendocrine cells, and added to the theme of a potential molecular

signaling mechanism of adhesion-GPCRs. Enteroendocrine cells function as specialized sensors of food components and nutrient metabolites. The cells are flask shaped with dense core secretory granules located at the base, from which peptide hormones are released, and with an apical microvillus–decorated sensory extension reaching the gut lumen.[24] Much attention has recently focused on the expression and function of 7TM receptors as chemosensors for metabolites, for example, of triglycerides—long chain fatty acids and 2-OG—and of complex carbohydrates generated by the gut microbiota—short chain fatty acids.[24,25] The enteroendocrine cells are, along with their neighboring enterocytes, renewed every week from pluripotent stem cells located at the bottom of the mucosal crypts.

Individual enteroendocrine cells are isolated and FACS purified after genetic labeling with GFP or RFP expressed under the control of promoters for gut hormones such as CCK.[26] Through quantitative polymerase chain reaction (qPCR) analysis, classical nutrient receptors were identified as being both highly expressed and highly enriched in the cells. Surprisingly, a number of adhesion-GPCRs were identified as being expressed at similar high levels in enteroendocrine cells as were specific nutrient metabolite receptors, for example, Celrs1, GPR128, Celsr3, CD97, and Lphn1. However, the majority of the adhesion-GPCRs were also highly expressed in the neighboring enterocytes, though a few were found to be both highly expressed and highly enriched in the enteroendocrine cells.

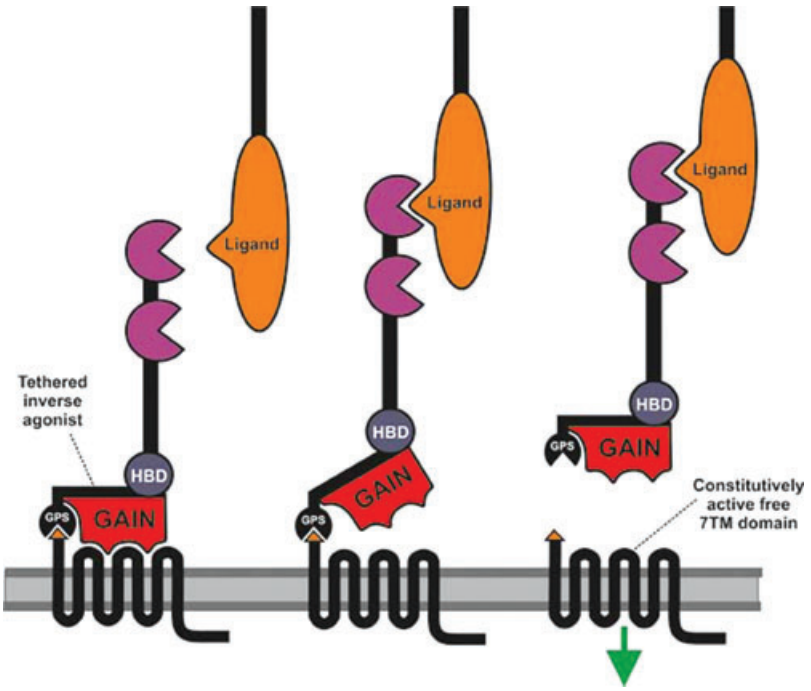

Figure 4. General model for molecular activation mechanism for adhesion-GPCRs. On the basis of molecular pharmacological studies on adhesion-GPCRs expressed on enteroendocrine cells, it is proposed that the large N-terminal extension in the full-length version of these receptors acts as a tethered inverse agonist, which, by binding to the 7TM domain, inhibits or silences the otherwise high constitutive activity of this domain. Upon binding of one or more of the far N-terminal ligand-binding domains (indicated in purple) to their macromolecular ligand, located either in *trans* on an opposing cell, in *cis* on the same cell, or in the intercellular matrix, the tethered inverse agonist (indicated in red) is removed from the 7TM domain, either partly (middle panel) or totally, which is possible in adhesion-GPCRs, provided that the GAIN/GPS domain–mediated autocleavage has occurred (right panel). Through this ligand binding–mediated process the free 7TM domain of the adhesion-GPCR will start signaling with its high constitutive activity, which, in this way, in fact, functions as a ligand/agonist-mediated signaling.

It has been rather unclear to what degree adhesion-GPCRs couple through classical G protein pathways. A number of the adhesion-GPCRs expressed in enteroendocrine cells were cloned and expressed heterologously in HEK293 cells using an optimized signal peptide construct to improve expression levels, though generally they displayed only minimal signaling. However, strong signaling through both G_q and G_i and, in particular, the SRE transcriptional activation pathway—that is, presumably through $G_{12/13}$—was observed when the receptors were expressed in an N-terminally truncated form in which only the small N-terminal segment from the autocleavage site to TM-I was intact. Thus, a general activation model for adhesion-GPCRs was proposed (see Fig. 4). In which the 7TM domain of the adhesion-GPCRs is highly constitutively active and the large N-terminal segment of the receptors functions as a tethered inverse agonist. That is, in the intact receptor, the N-terminal extension, or presumably the 3D-conserved GAIN domain, which after the autocleavage is noncovalently bound to the 7TM domain, will silence the constitutive signaling of this domain. It is proposed that binding of one or more of the far N-terminally located binding domains (which differ among the different adhesion-GPCRs) to a ligand attached on the neighboring cell or located on the same cell or in the intercellular matrix will lead to dissociation of the N-terminally tethered inverse agonist, which results in high constitutive signaling of the unbound 7TM domain left at the cell surface (Fig. 4).

This model is in agreement with a similar model suggested by the Hall and Xu groups on the basis of classical biochemical structure–function studies of GPR56, and by a model proposed by Langenhan and coworkers on the basis of *in vivo* structure–function studies performed in *C. elegans* with LAT-1/latrophilin.[13,17,27]

Shear stress–dependent downregulation of CD97 on circulating leukocytes by CD55

Jörg Hamann (University of Amsterdam) presented work on the ligand interactions of CD97, a prototypic adhesion-GPCR broadly expressed by hematopoietic and nonhematopoietic cells. CD97 interacts, through different regions in its extracellular subunit, with at least four other molecules: CD55, chondroitin sulfate B, $\alpha_5\beta_1$ integrin, and CD90/Thy-1. The ability of CD97 to engage with seemingly unrelated binding partners has triggered studies that aim to address the importance of individual ligands *in vivo* using the interaction with CD55 (Ref. 28) as a paradigm. These studies revealed that mice lacking either CD97 or CD55 had higher granulopoietic activity, resulting in increased numbers of circulating granulocytes.[29] Moreover, the absence of CD97 or CD55 reduced disease activity in two experimental models of arthritis.[30] In both cases, CD97 and CD55 knockout mice developed a highly similar phenotype; yet a causative relationship between the molecules could not be established.

Hamann described that circulating leukocytes from CD55-deficient mice express significantly increased levels of CD97. After adoptive transfer into of CD55-deficient leukocytes wild-type mice, CD97 expression on CD55-deficient leukocytes dropped to normal levels due to contact with CD55 expressed on wild-type leukocytes and stromal cells. Downregulation of CD97 occurred within minutes after first contact with CD55, involved both the extracellular and transmembrane subunit of the receptor, and correlated with an increase in plasma levels of soluble CD97. *In vitro*, downregulation of CD97 on CD55-deficient leukocytes cocultured with wild-type blood cells was strictly dependent on the shear stress from rigorous agitation of the cell cultures. *In vivo*, CD55-mediated downregulation of CD97 required intact circulation, as shown in experiments with wild-type recipient mice that were pretreated with heparin to prevent blood coagulation and then sacrificed immediately after adoptive transfer, followed by blood collection at later time points; transferred CD55-deficient leukocytes did not downregulate CD97 under these conditions. To test whether ligation by CD55 triggers CD97 signaling, CD55-deficient leukocytes were cocultured with wild-type blood cells. Notably, *de novo* ligation did not activate signaling molecules that recently were shown to be constitutively engaged by CD97 in cancer cells, such as ERK, PKB/Akt, and RhoA.[31]

Taken together, the findings presented confirm CD55 as a genuine binding partner of CD97 *in vivo*. They suggest that CD55 downregulates CD97 surface expression on circulating leukocytes by a process that requires physical forces but does not, based on current evidence, induce receptor signaling (Fig. 5). Regulation of CD97 expression by CD55 may prevent uncontrolled clustering of leukocytes

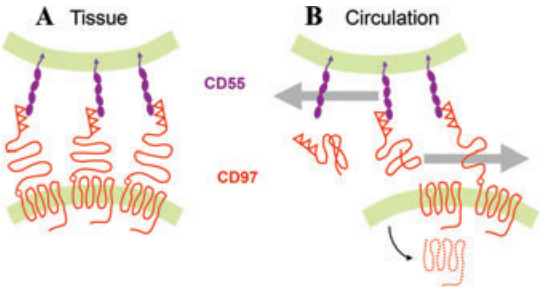

Figure 5. Consequences of the CD97–CD55 interaction *in vivo*. (A) In tissue, contacts between the adhesion-GPCR CD97 and its ligand CD55 likely facilitate cell adhesion. (B) In the circulation, CD97 expression is constantly downregulated by contact with CD55 on blood and stromal cells. This regulation process may prevent uncontrolled clustering of leukocytes in the blood stream, thereby restricting CD97–CD55 interaction-mediated adhesion to tissue sites.

due to homo- or heterotypic cellular contacts in the blood stream, thereby restricting CD97–CD55 interaction-mediated adhesion to tissue sites. The data support the hypothesis that adhesion-GPCRs are two-part entities with distinct roles for the extracellular and the seven-transmembrane subunits in cell adhesion and signaling, respectively.

Activation of the EMR2 receptor via ligation-induced translocation, and interaction of receptor subunits in lipid rafts activates macrophages

Hsi-Hsien Lin (Chang Gung University) analyzed the signaling mechanism of the adhesion-GPCR EMR2 in macrophages. Directed migration of phagocytes to infected sites is a critical step in innate immunity for pathogen elimination. Activated phagocytes clear invading pathogens by multiple mechanisms, including phagocytosis and release of proteases, antimicrobial peptides, and cytokine/chemokines. As a myeloid cell–restricted member of the adhesion-GPCR family, the EMR2 receptor has been shown previously to play a role in the cellular functions of innate immune cells.[32,33] Indeed, ligation of the EMR2 receptor not only can increase neutrophil adhesion and migration, but it can also augment the production of antimicrobial mediators.[32,33]

As with the majority of adhesion-GPCRs, EMR2 is posttranslationally modified by GPS autoproteolysis in the endoplasmic reticulum and cleaved into a large extracellular domain (α-subunit) and a seven-transmembrane domain (β-subunit).[10,34] To investigate the role of GPS autoproteolysis in mediating the cellular functions of adhesion-GPCRs and the mechanistic relevance of the receptor subunit interaction, Lin and coworkers first demonstrated that GPS proteolysis is necessary for EMR2-mediated cell migration. Next, the structural organization of EMR2 receptor subunits was examined. Surprisingly, two distinct receptor complexes were identified: one is a noncovalent α-β heterodimer, while the other consists of two independent receptor subunits with differential distribution in lipid raft microdomains. More specifically, the EMR2 α-subunit was shown to locate mostly in the nonraft regions, while the β-subunit was found in both the raft and nonraft regions. These data suggest that the two EMR2 receptor subunits do not always interact on the cell surface but behave, in part, as two independent molecules.[35]

Moreover, they showed that ligation of the EMR2 receptor by the α-subunit–specific 2A1 monoclonal antibody induces the translocation and colocalization of receptor subunits in lipid rafts. Such ligation activated the EMR2 receptor in macrophages, leading to the production of inflammatory cytokines such as IL-8 and TNF-α. Interestingly, cytokine production was inhibited when macrophages were treated with lipid raft disruptors lovastatin and filipin.[35] Thus, EMR2 receptor ligation–induced cytokine production seems to require intact lipid raft microdomains. Recently, preliminary data from Lin and coworkers indicated that EMR2 receptor activation via ligation of receptor subunits induces the activation of extracellular signal–regulated kinase (ERK), leading to macrophage activation. The induction of ERK phosphorylation in macrophages is 2A1 specific and dose and time dependent. Furthermore, ERK phosphorylation and cytokine production by macrophages via EMR2 receptor ligation was inhibited by U0126, an MEK inhibitor. Finally, supernatant of macrophages stimulated by 2A1-mediated EMR2 receptor ligation was found to stimulate human neutrophil activation, promote its transwell migration, and augment fMLP-induced ROS production. Taken together, these results demonstrate that the EMR2 receptor plays a critical role in innate immune functions (Fig. 6), and provides a paradigm for signal transduction within the adhesion-GPCR family.

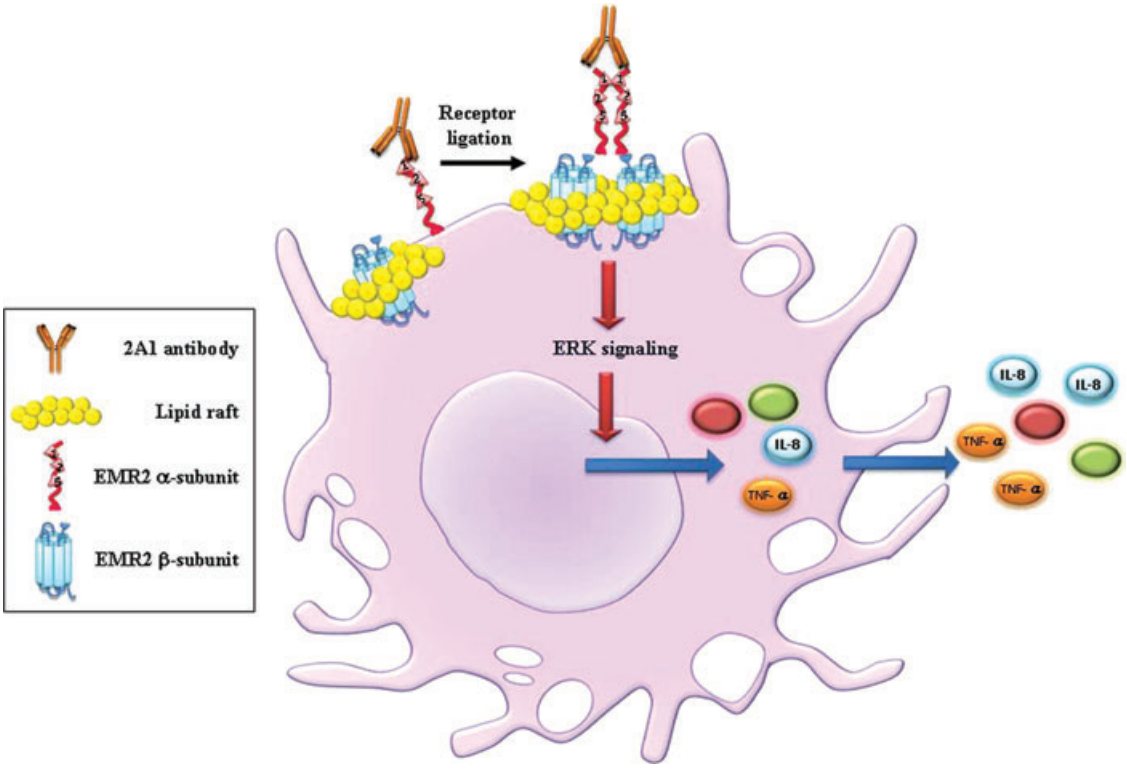

Figure 6. Activation of the EMR2 receptor in macrophages is mediated through the translocation and interaction of its two independent subunits in the lipid raft. The independent EMR2 α- and β-subunits are localized in the nonraft and lipid raft regions, respectively. Following receptor ligation by the α subunit–specific 2A1 mAb, the independent α-subunit translocates and reassociates with the β-subunit in the lipid raft region. Such interaction induces intracellular signaling via the MAPK pathway (mainly ERK1/2 phosphorylation), leading eventually to proinflammatory cytokine (IL-8, TNF-α) secretion.

Real-time monitoring of GPCR signaling in living cells

Davide Calebiro (University of Würzburg) concluded the workshop section on signaling of adhesion-GPCRs with an overview of *in vivo* imaging for GPCR signaling, which might be extended to adhesion-GPCRs one day. The approximately 1000 different GPCRs present on the surface of cells provide fundamental links between the extracellular environment and the intracellular milieu, allowing cells to respond and adapt to a wide variety of stimuli, such as hormones, neurotransmitters, light, and odors, as well as cell and matrix contacts. Whereas the basic molecular mechanisms of GPCR signaling have been elucidated, how such diverse stimuli are integrated via a few common signaling cascades while achieving highly specific responses is still poorly understood. In the last few years, Calebiro and colleagues developed a series of optical methods using fluorescence resonance energy transfer (FRET), which enables imaging GPCR activation and signaling directly in living cells.[36]

More recently, in order to analyze GPCR signaling under highly physiological conditions, Calebiro and colleagues have generated a transgenic mouse[37] with ubiquitous expression of a FRET sensor for cAMP[38] (Fig. 7A). This mouse has allowed them to study, among other aspects, the signals produced by the activation of a prototypical hormone receptor, that is, thyroid-stimulating hormone receptor (TSHR), directly in intact thyroid follicles (Fig. 7B). Unexpectedly, the results indicate that the TSHR, and possibly other GPCRs, can continue stimulating cAMP production even after internalization into the endosomal compartment, which leads to persistent signaling (Fig. 7C) and specific effects.[37,39] These data reveal new and important functions for receptor internalization in regulating GPCR-mediated responses. Calebiro and colleagues are currently using similar approaches with FRET sensors to further

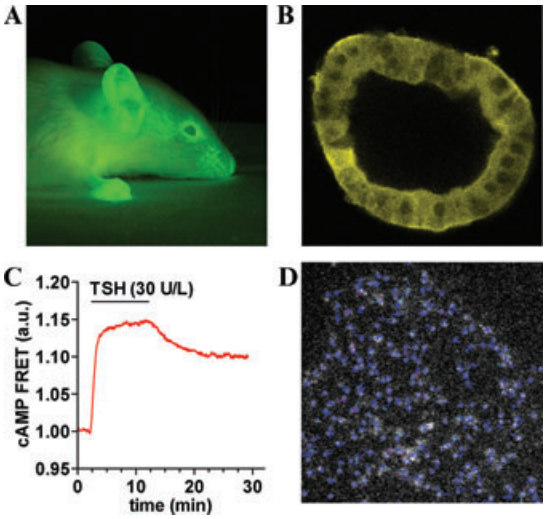

Figure 7. Real-time monitoring of GPCR signaling in living cells. (A) Transgenic mouse with ubiquitous expression of a FRET sensor for cAMP (Epac1-camps). (B) Confocal image of a thyroid follicle isolated from the cAMP reporter mouse. (C) Representative FRET trace obtained in a thyroid follicle, showing persistent cAMP elevations after transient TSH stimulation. (D) Single GPCRs on the surface of living cells visualized by TIRF microscopy.

explore this and other novel aspects of GPCR-cAMP signaling in fundamental physiological processes such as thyroid hormone production and female reproduction. Whereas these approaches allow a precise characterization of GPCR and second messenger signaling with high spatiotemporal resolution, a full characterization of GPCR signaling cascades will likely require observing the signals produced by the activation of a single receptor.

To achieve this goal, Calebiro and colleagues are developing new methods using labeling with small organic fluorophores and total internal reflection fluorescence (TIRF) microscopy, which allow visualizing signaling proteins at the surface of living cells with single-molecule sensitivity (Fig 7D). They are using these methods to monitor individual protein–protein interactions, such as those involved in ligand binding, receptor di-/oligomerization, or coupling to G proteins with high spatiotemporal resolution. Initial data suggest that GPCRs are targeted to different microdomains of the cell surface, where they are present in a dynamic equilibrium, with constant formation and dissociation of new receptor complexes. Taken together, these data provide novel insights into the complex dynamic events at the basis of the spatiotemporal compartmentalization of GPCR signaling cascades.

Adhesion-GPCRs in development

Accumulating evidence demonstrates that adhesion-GPCRs fulfill plentiful functions during the genesis of various organ systems. For some adhesion-GPCRs, these functions are well defined on a cell biological level, whereas other receptors remain to be placed in a physiological context.

Molecular and genetic analysis of Gpr126 in peripheral nerve development

Kelly Monk (Washington University School of Medicine) found a role for Gpr126 in myelin formation. Myelin is the multilayered glial membrane surrounding axons in the vertebrate nervous system. Myelin acts as an insulator that reduces the capacitance of the axonal membrane, thus increasing axon conduction velocity and allowing for fast processing speeds and efficient transmission of information over large distances. Myelin not only insulates axons: the glial cells that make myelin also protect and provide vital trophic support to neurons, and the importance of myelin is underscored in diseases in which it is disrupted, like multiple sclerosis and peripheral neuropathy. In the peripheral nervous system (PNS), Schwann cells produce myelin by iteratively wrapping their membranes around axons. Reciprocal signaling between axons and Schwann cells is required for proper myelination to occur, but the exact signaling mechanisms regulating myelination are poorly understood. An orphan adhesion-GPCR, Gpr126, is required for the initiation of myelination in zebrafish Schwann cells.[40] In the mouse, loss of *Gpr126* leads to complete amyelination in the PNS (Fig. 8) as well as multiple defects in peripheral nerves.[41] In zebrafish, Monk previously showed that forskolin treatment to elevate cAMP levels suppresses the mutant phenotype and restores myelination,[40] and they hypothesized that elevation of cAMP levels would similarly suppress mutant phenotypes in mouse mutants. Indeed, cAMP elevation or protein kinase A (PKA) activation in dorsal root ganglion explant cultures from *Gpr126* mutant mice rescued the myelin defects, providing further support that cAMP and PKA are involved in the Gpr126-mediated pathway initiating myelination. Although *Gpr126* is predominately

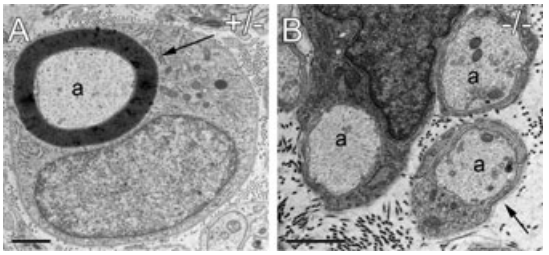

Figure 8. Gpr126 is essential for Schwann cell myelination. (A) An axon (a) is surrounded by myelin (arrow) in a postnatal day 12 *Gpr126*$^{+/-}$ sciatic nerve. (B) Schwann cells (arrow) ensheathe axons (a) in postnatal day 12 *Gpr126*$^{-/-}$ sciatic nerve, but do not make myelin. Scale bars = 1 μm.

expressed in Schwann cells, systemic *Gpr126* knockouts show pronounced axon loss. Therefore, Monk's group has begun to analyze conditional *Gpr126* mouse mutants, and their preliminary analysis suggests that Gpr126 is required autonomously in Schwann cells for myelination in mammals.

Discrepancies in Gpr126 knockout phenotypes

Felix Engel's laboratory (Max-Planck-Institute for Heart and Lung Research) performed a large-scale temporal mRNA expression analysis describing rat heart development from embryonic day (E) 11 to postnatal day 10 in an interval of 12 hours. This study identified the adhesion-GPCR *gpr126* as a gene that is transiently expressed during embryonic rat heart development. These data suggested that Gpr126 might be important for heart development. Moreover, it has recently been demonstrated that *gpr126* knockout mice exhibit a thinned myocardial wall. In addition, *gpr126* deletion has been described as embryonically lethal between E10.5 and E12.5 days post fertilization.[42] Preliminary data in zebrafish (in Engel's laboratory) further substantiated this hypothesis. By contrast, Monk *et al.* did not describe a heart phenotype or embryonic lethality in *gpr126* mutant zebrafish (*gpr126*^{st49}). Instead, they describe an ear phenotype and found that Gpr126 is essential for Schwann cells to initiate myelination.[40] What could explain the discrepancies between the GPR126 knockout mouse and knockdown or mutant zebrafish phenotypes?

Little is known about Gpr126. As for most adhesion-GPCRs there is no identified natural or synthetic ligand of Gpr126. Gpr126 contains a seven-transmembrane domain (7TM), here called the C-terminal fragment (CTF), and an extracellular domain containing a CUB (complement, Uegf, Bmp1) domain and a pentraxin (PTX) domain,[43] here called the N-terminal fragment (NTF). The NTF and the CTF are linked via a GPS-containing stalk region, which is highly conserved among adhesion-GPCRs. Adhesion-GPCRs appear to be cleaved at the GPS immediately after biosynthesis, but NTF and CTF are generally believed to stay noncovalently associated after cleavage and to be expressed on the cell membrane as a heterodimer. Importantly, Volynski *et al.* showed that the NTF of the adhesion-GPCR latrophilin can be self-anchored to the cell membrane independently of the CTF.[11] Currently, it is unclear whether NTFs of other adhesion-GPCRs can exist independently and what physiological importance they have. However, Moriguchi *et al.* have previously demonstrated that Gpr126 (DREG) is also cleaved and that a small fragment is secreted.[44] Interestingly, the mutation in the *gpr126*^{st49} fish introduces a stop codon just before the GPS domain.[40] Thus, it is likely that *gpr126*^{st49} mutant fish might express a functional NTF. Therefore, Engel hypothesizes that the NTF of Gpr126 functions independently of its CTF and that the NTF, but not the CTF, is required for the developing heart. To test this hypothesis Engel and colleagues are performing selective knockdowns of CTF or the entire *gpr126* in zebrafish. Moreover, they will analyze whether overexpression of NTF in Gpr126 morphants can rescue the heart but not the ear phenotype. Finally, they are in process to generate conditional knockout mice to better determine cell type–specific functions of Gpr126.

Role of Celsr1–3 cadherins in planar cell polarity and ependymal development

André Goffinet (Université Catholique de Louvain) researches the CELSR group of adhesion-GPCRs. Cadherin EGF LAG seven-pass G-type receptors 1, 2, and 3 (Celsr1–3) form a family of three atypical cadherins with multiple functions in epithelia and the nervous system. During the last few years evidence has accumulated for important and distinct roles of Celsr1–3, as well as other genes, such as Frizzled3 (Fzd3), in planar cell polarity (PCP) and brain development and maintenance.[45,46] Celsr1–3 harbor large ectodomains, composed of nine N-terminal cadherin repeats, EGF-like domains, laminin G repeats, one hormone receptor motif, and

a potential GPS. This is followed by seven transmembrane domains and a cytoplasmic tail. Like their fruit fly ortholog Flamingo (*Fmi*), Celsr1–3 are thought to work in interaction with other core PCP proteins such as Frizzled (particularly Fzd3 and Fzd6 in mammals), Van Gogh (Vangl1–2), Dishevelled (Dvl1–3), and Prickle (Pk1–4).

Observations of constitutive and conditional Celsr2 and 3 mutant mice uncovered important functions of these proteins during ependymal development. In double mutant animals, a severe hydrocephalus develops rapidly during the early postnatal period (P), leading to death around P8–P10. Studies with scanning and transmission microscopy and immunohistochemistry found prominent abnormalities of ciliogenesis in mutants. Basal bodies remained frequently embedded in the subapical cytoplasm and failed to become aligned normally (rotational polarity defect). As a result, many ependymal cells are completely devoid of cilia and the circulation of the CSF is severely impaired, leading to lethal hydrocephalus.[46]

More recently, Goffinet has studied the role of Celsr1 during ependymal development using conditional and floxed Celsr1[47] and Vangl2[48] mutant alleles, showing that they are distinct from that of Celsr2 and 3. Whereas cilia appear at the normal time and are of normal length, in Celsr1 mutant mice there is a clear defect in translational polarity, in that cilia tufts are not consistently displaced rostrally, as in normal animals; a translational polarity defect was seen even at P0, when ependymal cells had one nonmotile cilium, in Celsr1 mutants and in Fzd3 and Vangl2 mutants. Rotational polarization of basal bodies (BB) was studied using double labeling with gamma-catenin for BB and phosphocatenin, which labels an area adjacent to BB, opposite the basal foot. Rotational polarization of BB was found to be similarly defective in Celsr1 and Vangl2 mutants (Fzd3 mutants die at P0 and cannot be studied). In addition, the mean vectors of translational and rotational polarization are not aligned in mutant ependymal cells, unlike normal ependymal cells.

Goffinet's data show that Celsr1 regulates translational and rotational polarity of ependymal cells from the time that they are generated to the adult stage. Celsr1 works together with Vangl2 and Fzd3 in this process. Such PCP regulation in the ependymal epithelium is complementary to the role of Celsr2 and 3, which regulate ependymal differentiation and ciliogenesis.

Adhesion-GPCR Celsr1 in the complex morphogenesis of mammalian organ primordia

Caroline Formstone (King's College London) reported about additional functions of Celsr-like adhesion-GPCRs. Mice with disrupted core PCP component function die at birth owing to catastrophic developmental defects in neural tube closure[49] and lung branching.[50] Defects in the development of other organ systems are also apparent including the epidermis.[51] The adhesion-GPCR Flamingo plays a central role in the local transmission of PCP information among neighboring cells. Of three Flamingo homologues in mammals, Celsr1 is predominantly found in epithelial precursors within organ primordia. Several studies strongly indicate a major role for Celsr1 in the coordination of PCP during mammalian organ development,[47,51] but how it functions to coordinate epithelial morphogenesis is unclear. Formstone's recent data suggest that Celsr1 protein exhibits a differential distribution along the apicobasal axis of some epithelia. In particular, Celsr1 exhibits a novel enrichment to the basal membrane of neuroepithelial precursors and lung tubules.[52,53] Studies on how differential Celsr1 protein distribution links to its function in tissue morphogenesis and whether Celsr1 protein at the epithelial basal membrane elicits PCP signaling will provide insight into the complex roles of Celsr1 in mammalian organ development.

CD97 overexpression induces a megaintestine

Interestingly, CD97 is also implicated in intestinal development, as presented by Gabriela Aust (Universtiy of Leipzig). Adhesion-GPCRs are involved in adhesion, guidance, and positioning of cells. CD97, in contrast to the other EGF-TM7 adhesion-GPCR subfamily members restricted to immune cells, is present in normal and malignant epithelial cells. In normal human intestine, CD97 is located in enterocytic cell–cell contacts[54] and, in the cytoplasm, shows an expression gradient along the crypt–villus axis.

To understand the role of CD97 in intestinal physiology, Aust *et al.* generated transgenic Tg(villin-CD97) mice.[54] Unexpectedly, overexpression of CD97 resulted in upper megaintestine, depending

on the CD97 cDNA copy number integrated. Intestinal enlargement involved an increase in length, diameter, and weight. Remarkably, the megaintestine phenotype develops with normal microscopic morphology, and thereby clearly differs from existing megaintestine models in which intestinal enlargement is often accompanied by dramatic morphological changes.

The megaintestine phenotype is acquired after birth before weaning, which makes this a unique model for investigating the mechanisms underlying postnatal expansion of the mammalian small intestine by way of two consecutive growth patterns: (1) cylindrical growth in length and diameter without alteration of microscopic morphology (as seen in the Tg(villin-CD97) mice), and (2) luminal growth with amplification of the internal surface area.[55]

Notably and in accordance with a cylindrical growth pattern, suckling but not adult Tg(villin-CD97) mice showed more crypt fission compared with wild-type mice. Consistently, acquisition of the megaintestine was independent of altered cell lineage determination, Wnt signaling, and an increase of intestinal stem cell markers. Suckling Tg(villin-CD97) pups developed the phenotype independent of the genotype of the feeding dam, thus excluding regulation of a milk growth factor by CD97. Most likely, CD97 regulates the binding or signaling of an intestinal receptor for a milk constituent.

The Tg(villin-CD97) mice provide new evidence supporting the conclusion that adhesion-GPCRs have distinct functions that may depend on the cellular context in which a given receptor is expressed. Tg(villin-CD97) mice not expressing CD55 that binds to the extracellular EGF-like domains of CD97 also developed a megaintestine, suggesting that the adhesive extracellular part is not necessary for phenotype induction. By contrast, mice overexpressing a truncated CD97 with only the first two transmembrane helices did not develop a megaintestine, which implies signaling through CD97 in phenotype induction.

Overall, these transgenic mice provide suitable models to uncover and understand functions of adhesion GPCRs in normal epithelial cells.

Neurobiological roles of adhesion-GPCRs

The connection between adhesion-GPCRs and neural function was postulated early on. Some groups reported about their ongoing efforts to pinpoint the exact relationship between these receptors and the properties of this highly specialized cell type.

Drosophila *synapses as an* in vivo *model to study structure–function relationships of latrophilin*

Tobias Langenhan and Robert Kittel (University of Würzburg) introduced the fruit fly *Drosophila melanogaster* as a new *in vivo* model for research on evolutionarily conserved adhesion-GPCRs of the latrophilin group, which is extendable to the Flamingo/CELSR group. Latrophilins have been implicated in the control of synaptic transmission as well as planar cell polarity, raising the questions of whether and how these phenomena are interlinked.[15,16,56] Thus far, models in which cell polarity and neuronal exocytosis could be tested at the same time and in the same cell type have been lacking in the adhesion-GPCR field. The versatile model system of *Drosophila* includes high-throughput transgenesis with single copy integration, homologous recombination for knockout/knockin studies of selected target genes, cell- and time-specific transgene expression through binary expression systems, and a vast arsenal of allelic variants covering the entire genome for genetic interaction studies. In particular, the fruit fly larva possesses a well-defined synaptic contact, the neuromuscular junction (NMJ), ideally suited for investigating adhesion-GPCR expression and function with biochemical, imaging, and electrophysiological methods. Langenhan and Kittel presented preliminary data indicating that latrophilin/dCIRL is resident at the NMJ, and that its removal by mutation or RNA interference causes changes in the molecular, structural, and functional properties of this synapse type. After full characterization of the phenotypic profile due to *dCirl* deficiency, Langenhan and Kittel will use the NMJ as an *in vivo* platform to test modified *dCirl* variants and correlate molecular lesions in the receptor with functional consequences on a cell biological level.

Transsynaptic interaction between presynaptic latrophilin and postsynaptic Lasso

Yuri Ushkaryov (University of Kent) is interested in the function of mammalian latrophilins and presented his latest data. Latrophilin 1 (LPH1),[57] a neuronal adhesion G-protein–coupled receptor that binds α-latrotoxin, is implicated in control of presynaptic Ca^{2+} and in the modulation of

neurotransmitter release.[58,59] To understand the molecular mechanisms of these physiological functions, Ushkaryov's group isolated the endogenous ligand of LPH1, Lasso.[60] This protein is a splice variant of teneurin-2. Teneurins are brain-specific, orphan, cell surface receptors with functions in neuronal pathfinding and synaptogenesis. Ushkaryov's data indicate that LPH1, located on presynaptic terminals, forms strong and specific transsynaptic complexes with Lasso, which is found on postsynaptic spines. This interaction is not only structural but also functional: soluble fragments of Lasso induce intracellular Ca^{2+} signals upon binding to LPH1 in presynaptic boutons of cultured hippocampal neurons and in nonneuronal cells expressing exogenous LPH1. Furthermore, the LPH1–Lasso complexes play an important role in synaptic development and activity. Thus, Lasso fragments acting via LPH1 strongly increase the rate of spontaneous exocytosis in mouse neuromuscular junctions.[61] LPH1 expressed on nonneuronal cells induces postsynaptic differentiation in cocultured hippocampal neurons.[60] On the other hand, while synapses in which the interaction between LPH1 and Lasso is inhibited, appear morphologically normal, they remain physiologically silent.[61] Taken together, the data from the Ushkaryov group indicate that while the transsynaptic interaction of LPH1 and Lasso is not necessary for the initial establishment of central synapses, it participates in presynaptic Ca^{2+} control and is required for functional maturation of presynaptic nerve terminals.

The very large G PCR Vlgr1b/GPR98 – a key component of the Usher syndrome protein networks

Uwe Wolfrum (University of Mainz) described the role of the very large G protein–coupled receptor-1 (VLGR1) in the inner ear and in retinal biology. VLGR1, also known as MASS1 or GPR98, has a molecular weight of up to ∼700 kDa and is by far the largest GPCR and the largest cell surface protein known to date.[62] The large ectodomain of the largest splice variant VLGR1b contains several repeated motifs, including calcium binding, Calx-β repeats, and seven copies of an epitempin repeat. It is linked to the 7TM moiety via a proteolytic site (GPS) containing a region typical for adhesion-GPCRs. The short intracellular C-terminus contains a consensus PDZ binding motif, suggesting interactions with cellular scaffold proteins. In the absence of any known ligand VLGR1/GPR98 is one of the few adhesion-GPCRs in which mutations are disease relevant. VLGR1/GPR98 defects are thought to be associated with epilepsy. Mouse *vlgr1* mutants are characterized by the susceptibility to audiogenic seizures and to the development of sensoneuronal defects, namely hearing impairment and visual dysfunction.[63,64] Mutations in the human VLGR1/GPR98 gene cause Usher syndrome (USH) type 2C.[65]

Human USH is the most common form of combined hereditary deaf-blindness. Three clinical subtypes (USH1–3) are differentiated on the basis of severity, age of onset, and progression of the symptom.[65] Wolfrum and others have identified VLGR1/GPR98 as a component of USH protein networks in inner ear hair cells and retinal photoreceptor cells. In hair cells VLGR1/GPR98 is part of the ankle link complex essential for the formation of the ankle links between the membranes of neighboring stereocilia and thereby for the correct development of the mechanosensitive hair

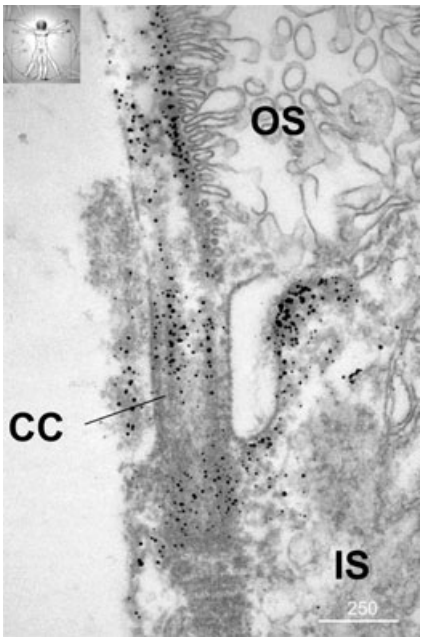

Figure 9. **Immunoelectronmicroscopy localization of VLGR1/GPR98 in a photoreceptor cell of the human retina. VLGR1/GPR98 is located in the connecting cilium (CC) and periciliary region of the rod cell inner segment (IS), as well as along the axoneme of the outer segment (OS). Bar = 250 nm.**

bundles.[63] In photoreceptor cells VLGR1/GPR98 is a component of the periciliary USH protein network, which is crucial for cargo transport to the photoreceptor cilium (Fig. 9).[64] In this periciliary network, VLGR1/GPR98 is required for the assembly of fibrous links communicating between the membranes of the inner segment and the connecting cilium of photoreceptor cells. In both sensory systems, VLGR1/GPR98 is additionally found at synapses, where it is specifically localized in postsynapses of the dendritic tips of retinal bipolar cells and in spiral ganglion neuritis, respectively.[66]

The identification of further components of these protein networks, the decipherment of the downstream cellular signaling pathway, and knowledge about ligands of VLGR1/GPR98 will lead not only to a better understanding of protein function, but will also enlighten the pathomechanisms underlying the USH disease, which is a necessary prerequisite for the development of future therapy concepts.

GPR56-dependent development of the frontal cerebral cortex

Xianhua Piao (Harvard Medical School) investigated the function of the adhesion-GPCR GPR56 in neural development. Although the human cerebral cortex is subdivided into dozens of specific areas with divergent functions, the genetic mechanisms underlying the regional development of the cerebral cortex are very poorly understood. One approach to studying the mechanisms of cortical specification is genetic analysis of inherited conditions in which specific regions of the cortex are preferentially perturbed. Bilateral frontoparietal polymicrogyria (BFPP), a recessively inherited genetic disorder of human cerebral cortical development, shows severely abnormal architecture in the frontal lobes, with milder involvement of the posterior parts of the cortex (Fig. 10A and B).[67] Linkage analysis and positional cloning in a cohort of 22 BFPP patients revealed that GPR56 is the causative gene of BFPP.[68] This discovery demonstrated a novel signaling pathway in the developmental regulation of regionalization of the cerebral cortex.

Unraveling the ligand of GPR56 is the first step in revealing the signaling pathway of GPR56. A receptor affinity probe *in situ* approach demonstrated that the putative ligand of GPR56 is expressed in the meninges and pial basement membrane (BM). Subsequent proteomic and genetic studies identified collagen III as the endogenous ligand of GPR56 in the developing brain.[69] Upon binding to collagen III, GPR56 activates RhoA via coupling to Gα12/13. RhoA activation has been shown to regulate cell migration. To study GPR56-mediated RhoA activation on neuronal cell migration, an *in vitro* neurosphere migration assay was performed. The presence of collagen III inhibits neuronal migration

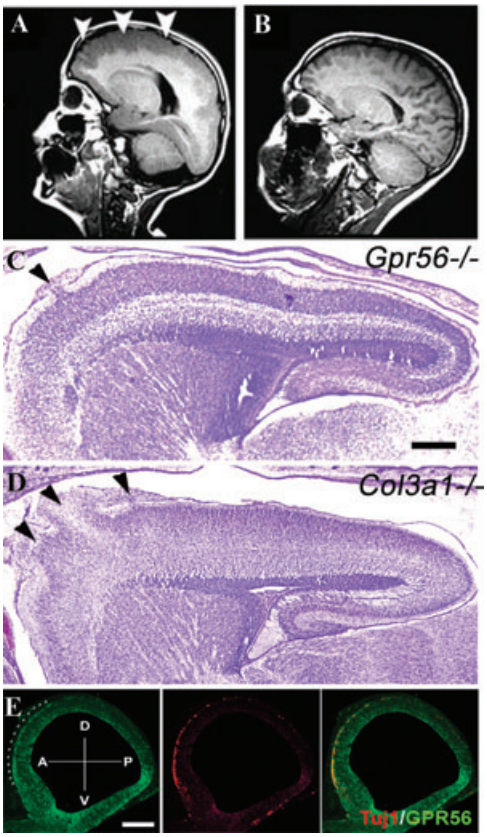

Figure 10. The gradient expression of GPR56 in the preplate correlates with the anatomical distribution of cortical defects associated with mutations in GPR56 and Col3a1. Compared to the MRI of a normal brain (B), multiple small gyri with a scalloped appearance of the cortical – white matter junction were predominantly seen in the frontal cortex in a BFPP brain (white arrowheads in A). This regional deformation of the cerebral cortex (black arrowheads) was recapitulated in Gpr56 (C) as well as in Col3a1 (D) knockout mouse brains. Interestingly, the restricted expression of GPR56 (green) at the basal surface of embryonic day 10.5 mouse brain (outlined by white dotted line in E, left panel) matches the anatomical distribution of the cortical defects seen in both humans and mice when GPR56 or its ligand collagen III is deleted. A–P and D–V axes are shown in E. Abbreviations: A, anterior; P, posterior; D, dorsal; V, ventral. This figure is adapted from previous publications as follows: A and B from Ref. 68; C from Ref. 69; E from Ref. 72.

in a GPR56-dependent fashion. This observation was further confirmed in *Gpr56* and *Col3a1* knockout mouse brains (Fig. 10C and D).[70,71] Taken together, Piao's data indicate that the interaction of GPR56 and its ligand collagen III inhibits migrating neurons from breaking through the pial BM, thus conveying a positional cue during cortical development.

Because the regulation of rostral cortical development by GPR56 signaling could be accomplished by regional expression of either GPR56 or its ligand collagen III, Piao and coworkers studied the expression profile of both proteins in the developing cortex. Immunohistochemistry of collagen III on sagittal sections of mouse embryonic brains ranging in age from E10.5 to E11.5 did not reveal an expression gradient of collagen III during these developmental stages.[72] In contrast, an anterior-to-posterior gradient of GPR56 protein expression was found on the basal surface of the neocortex in both E10.5 and E11.5 brains (Fig. 10E), but dissipated by E12.5. This finding is particularly interesting, as the change in the expression pattern occurs in the region where preplate neurons reside.

During cerebral cortical development first-born neurons form the preplate directly beneath the pial BM and function as a framework for further development of the cortex. However, the molecular mechanism underlying the function of the preplate neurons remains largely unknown. The fact that a gradient expression of GPR56 in preplate neurons matches the regional cortical defects associated with loss of GPR56, or its ligand collagen III, (Fig. 10) suggests that a novel receptor–ligand pair is responsible for mediating the interaction between preplate neurons and the pial BM, thus defining the boundary between the neocortex and the meninges, while providing a framework for the developing cortex. Further testing of this hypothesis will undoubtedly advance our understanding of the molecular mechanisms underlying how preplate neurons regulate cortical development.

Emerging roles of adhesion-GPCRs in disease

It becomes increasingly clear that adhesion-GPCR dysfunction is involved in several human conditions. Research into these pathological states not only helps elucidate molecular breaking points of adhesion-GPCR signals, but also assists in developing remedies and direct pharmacological efforts to counteract adhesion-GPCR–dependent diseases. At the workshop, the roles of adhesion-GPCRs in tumorigenesis were discussed.

GPR56 and cancer

Lei Xu (University of Rochester Medical Center) presented studies from her lab on the roles of the adhesion-GPCR GPR56 in cancer progression. She and colleagues previously showed that GPR56 is downregulated in highly metastatic melanoma cells and that its re-expression led to inhibition of metastasis and melanoma growth.[73] Insights on how this might occur came from the identification of a putative ligand of GPR56, TG2.[73] TG2, also called tissue transglutaminase, is a crosslinking enzyme in the extracellular matrix (ECM) that modulates ECM biophysical properties.[74] TG2 also possesses crosslinking-independent functions and interacts with integrins and ECM proteins to regulate cell adhesion.[75] The signaling mechanisms of GPR56, as well as whether and how TG2–GPR56 interaction regulates melanoma progression, are outstanding questions for which progress was then discussed.

Initial lines of investigation revealed that angiogenesis is impaired in tumors expressing high levels of GPR56.[27] Angiogenesis, the process of nascent blood vessel formation, is essential for sustained tumor growth. Overexpression of GPR56 in the melanoma cell line MC-1 inhibited production of vascular endothelial growth factor (VEGF), a major contributor to angiogenesis, and resulted in a decrease in angiogenesis and tumor growth. In contrast, deletion of a ~70 aa serine threonine proline–rich (STP) segment in the α-subunit of GPR56 resulted in a significant elevation of VEGF production and enhanced angiogenesis and melanoma growth. The opposite effects of GPR56 and ΔSTP-GPR56 in melanoma cells indicate that the seven transmembrane domains of GPR56 (GPR56β) might exist in different activation states, and that these states might be modulated by the α-subunit and/or its binding partners (Fig. 11). Consistent with this model, addition of purified GPR56α was sufficient to inhibit VEGF production from MC-1 cells expressing GPR56β.[27]

Since the STP segment in GPR56 was both necessary and sufficient for binding to TG2, the opposing effects of ΔSTP-GPR56 and full-length GPR56 on

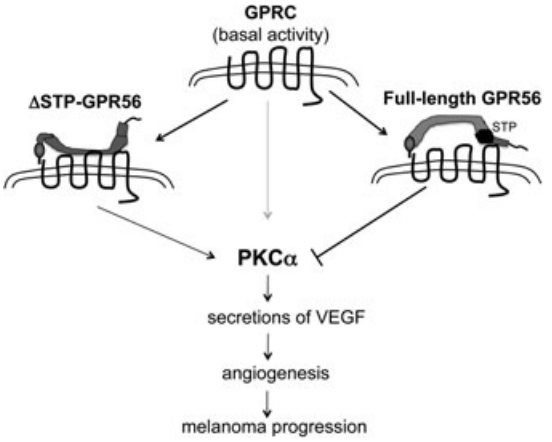

Figure 11. Different activation states of GPR56.

VEGF production implied that the GPR56–TG2 interaction was required for regulation of VEGF production by GPR56. Nevertheless, in contrast to the effect of ΔSTP-GPR56, knockdown of TG2 by shRNAs did not result in elevated VEGF production, indicating that the roles of the TG2–GPR56 interaction in melanomas might be more complex. To investigate this further, researchers analyzed growth of melanoma cells expressing GPR56 cDNA or shRNAs in immunodeficient $Tg2^{-/-}$ mice. Preliminary data suggested an unexpected antagonistic relationship between GPR56 and TG2 in melanomas: while GPR56 inhibited melanoma growth, TG2 promoted it (work in progress). Furthermore, the absence of TG2 abolished the effects of GPR56 on melanoma growth, indicating that TG2 might act downstream of GPR56, i.e., GPR56 might inhibit the tumor-promoting role of TG2. The mechanisms of this antagonism were revealed through a series of immunohistochemical and biochemical analyses. GPR56 expression was found to induce changes in the distribution pattern of TG2 in melanomas, probably due to a downregulation of TG2 in the ECM of GPR56-expressing melanoma cells. This downregulation was confirmed by *in vitro* studies that showed that the extracellular TG2 was internalized by GPR56 and subsequently degraded intracellularly in a lysosome-dependent manner.

Aberrant adhesion-GPCR expression in breast cancer—a potential role in metastasis?

Martin Stacey (University of Leeds) reported on the connection between adhesion-GPCRs and metastasis development. EGF-TM7 adhesion-GPCRs are predominantly expressed on leukocytes, including macrophages, dendritic cells, and neutrophils. Through the use of a stimulating antibody (2A1), Stacey and colleagues have shown that ligation of the human-restricted EGF-TM7 receptor EMR2 results in the enhanced activation of human neutrophils. Data demonstrate an increase in reactive oxygen species generation, degranulation (myeloperoxidase), and surface marker expression (CD11b and L-selectin shedding) upon ligation by 2A1. Furthermore, EMR2 transfectants displayed an increase in *in vitro* cell migration and invasion.[33] Truncations of the transmembrane domains and mutants preventing cleavage at the GPS site demonstrated the requirement of an intact transmembrane domain and receptor processing to elicit cell signaling,[33] showing that signaling is indeed required for EMR2 function. Overall, the data suggest an important role in the activation and migration of human leukocytes. Interestingly despite its leukocyte-restricted profile of EMR2, Stacey and colleagues show that mRNA and protein are aberrantly present in epithelial cells of breast cancer tissue.[76] Moreover receptor isoform expression is similar to that seen in neutrophils and macrophages, suggesting a potential hijacking of the normal function of EMR2 for tumor activation, migration, and progression. qPCR and flow cytometric analysis of EMR2-transfected breast cancer cell lines demonstrated increased expression of the epithelial-to-mesenchymal transcription factors snail and twist and decreased expression of the epithelial marker E-cadherin. Further, potential roles of EMR2 in tumor progression are to be investigated. Tools for targeting of EGF-TM7 receptors have been generated through the use of recombinant antibody fragments; for example, scFv and diabodies of antibodies against EMR2 and F4/80 have been cloned and fused to either toxins or model peptides. These reagents will be used in future depletion studies and in the receptor-specific targeting of antigens to leukocyte subsets.

The potential role of CD97 in the biology of acute myeloid leukemia

Manja Wobus (University Hospital, Dresden) found that the adhesion-GPCR CD97 is involved in acute myeloid leukemia (AML). AML cells home to a specified region of the bone marrow (BM), where they interact with stromal components, including extracellular matrix proteins, glycosaminoglycans,

and stromal cells, by which they derive proliferative and growth inhibitory signals. Different receptors, for example, VLA- (very late antigen-) 4, CXCR4, and CD44, described to play a critical role in normal stem cell homing, also appear to be paramount to the homing of AML cells to, or retention within, the bone marrow.[77]

CD97 is differentially expressed in murine hematopoietic stem and progenitor cells (HSPCs),[78] but nothing is known about its expression in human hematopoietic progenitor cells. Wobus hypothesizes that CD97 is involved in AML progression and manifestation, potentially by interaction with its recently described ligand CD90/Thy-1, which is expressed by nonhematopoietic cells in the BM microenvironment.[79] They therefore initiated a comprehensive investigation of *de novo* AML samples and correlated the CD97 expression to clinically important parameters, such as NPM1 and FLT3 mutations.[80] The AML cell lines MV4–11 and EOL-1, as well as $CD34^+$ HSPCs, were used to study CD97 expression and regulation *in vitro*.

Compared to BM blasts of healthy donors, they detected significantly higher CD97 expression (mean fluorescence intensity, MFI) in 42% of AML samples. Patients with CD97 expression above the mean on leukemic blasts also showed increased expression of the molecule within the residual granulo- and monopoiesis. Of note, higher CD97 expression was accompanied by a significantly higher BM blast count (75% vs. 53%, $P < 0.001$). Interestingly, elevated CD97 expression was associated with mutations in NPM1 (46% vs. 18%, $P = 0.003$) and FLT3 genes (39% vs. 7%, $P < 0.001$), as well as lower CD34 expression (46% vs. 81%, $P < 0.001$). Furthermore, no AML1/ETO or CBFb/MYH11 fusion genes were detectable in $CD97^+$ AML versus 6% in CD97-AML.

Figure 12. (Top) Participants of the Adhesion-GPCR Workshop in front of the Residence Palace, Würzburg. Not shown: Demet Araç, Robert Kittel, Alexander Petrenko, Helgi Schiöth, Thue Schwartz, and Yuri Ushkaryov. (Bottom) Participants of the Adhesion-GPCR Workshop in the lecture hall of the Institute of Physiology, Würzburg. Not shown: Demet Araç, Davide Calebiro, Robert Kittel, Tobias Langenhan, Manja Wobus, and Lei Xu.

In vitro, Wobus detected lower CD97 expression levels in primary $CD34^+$ HSPC compared to the AML cell lines. Of note, in FLT3-ITD mutated MV4–11 cells, CD97 was expressed significantly higher. Treatment of this cell line with different tyrosine kinase inhibitors resulted in a decreased CD97 expression. The lower CD97 expression levels correlated with inhibition of the spontaneous migratory capacity. By using a dicer-substrate 27-mer duplex targeting CD97 in MV4–11 cells, Wobus knocked down expression to about 45%, which correlated with decreased transwell migration.

In summary, Wobus provides the first evidence of higher CD97 expression in AML cells compared to normal $CD34^+$ hematopoietic cells *in vivo* and *in vitro,* which correlates with FLT3-ITD mutation. In ongoing studies the underlying regulatory mechanisms will be investigated. The possible impact of CD97 as well as other molecules of that receptor family on AML biology and clinical outcome will be evaluated in a larger patient cohort.

Conclusions

The 6th International Adhesion-GPCR Workshop (photographs of workshop participants shown in Fig. 12) has yielded intricate details regarding the molecular faculty of these peculiar membrane proteins. The collective efforts of all the labs now allow us for the first time to speculate on the events from stimulus reception by an adhesion-GPCR via transduction to signal generation inside the cell, which is a remarkable milestone in the history of research on adhesion-GPCRs. The biological setting, the unusual important signaling route, became defined more clearly, allowing novel glimpses of adhesion-GPCR function and dysfunction. It is clear that interdisciplinarity and open scientific exchange is an important prerequisite and driving force to achieving this current level of understanding.

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Conflicts of interest

The authors declare no conflicts of interest.

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Appendix

6th International Adhesion-GPCR Workshop

6–8 September 2012 – Würzburg – Germany

Scientific program

Thursday, Sep 6 2012

9:00 – 9:10
Opening remarks
Tobias Langenhan, University of Würzburg

Session A – Structural hallmarks of Adhesion-GPCR

9:10 – 9:35
The GPS motif: 15 years of studies
Alexander Petrenko, Russian Academy of Sciences, Moscow
9:35 – 10:00
A Novel Evolutionarily Conserved Domain of Cell-Adhesion GPCRs Mediates Autoproteolysis
Demet Araç-Ozkan, Stanford University
10:00 – 10:25
Structural insights into the adhesion-GPCR CD97
Martin Stacey, University of Leeds
10:25 – 10:45
Coffee break

Session B – Neurobiological roles of Adhesion-GPCR

10:45 – 11:10
Latrophilin receptors regulate presynaptic transmitter release
Tobias Langenhan, University of Würzburg
11:10 – 11:35
High-affinity functional trans-synaptic receptor pairs between presynaptic latrophilin and postsynaptic Lasso (teneurin-2)
Yuri Ushkaryov, University of Kent
11:35 – 12:00
GPR56-dependent development of the frontal cerebral cortex
Xianhua Piao, Harvard Medical School
12:00 – 14:00
Lunch break

Session C – Neurobiological roles of Adhesion-GPCR (continued)

14:00 – 14:25
GPR56, together with α3β1 Integrin, Regulates Cortical Lamination
Kathleen Singer, Harvard Medical School
14:25 – 14:50
Role of Celsr1–3 cadherins in planar cell polarity and brain development
André Goffinet, University of Louvain
14:50 – 15:15
The very large G protein coupled receptor Vlgr1b/GPR98 as a key component of the Usher syndrome protein networks in the inner ear and the retina
Uwe Wolfrum, University of Mainz
15:15 – 15:40
Molecular and genetic analysis of Gpr126 in peripheral nerve development
Kelly Monk, Washington University School of Medicine, St. Louis
15:40 – 16:30
Coffee break
19:00
Evening program

Friday, Sep 7 2012

Session D – Adhesion-GPCR in development

9:00 – 9:25
Basal enrichment of Celsr1 protein within epithelia: novel function or apico-basal dependent planar cell polarity (PCP) signalling?
Caroline Formstone, King's College London
9:25 – 9:50
Knockdown of the orphan G protein-coupled receptor 126 influences ventricular morphogenesis and heart function in zebrafish and mice
Felix Engel, Max-Planck-Institute Bad Nauheim
9:50 – 10:15
Mice constitutively overexpressing CD97 in enterocytes develop a megaintestine without alterations in histology and cell fate decision
Gabriela Aust, University of Leipzig
10:15 – 10:45
Coffee break

Session E – Adhesion-GPCR in tumor biology

10:45 – 11:10
Roles of GPR56 and TG2 during Melanoma Progression
Lei Xu, Rochester School of Medicine
11:10 – 11:35
Molecular characterization of the interaction of GPR56 and a novel ligand
Hsi-Hsien Lin, Chang Gung University
11:35 – 12:00
The expression of the EGF-TM7 receptor CD97 is higher in CD34-negative and NPM1/FLT3-ITD mutated AML
Manja Wobus, University of Dresden

12:00 – 14:00
Lunch break

Session F – Mechanisms of signal transduction of Adhesion-GPCR

14:00 – 14:25
Shear stress-dependent downregulation of the adhesion-GPCR CD97 on circulating leukocytes upon contact with its ligand CD55
Jörg Hamann, University of Amsterdam
14:25 – 14:50
Insights into the molecular function of latrophilins – logic of adhesion-GPCR signalling
Simone Prömel, University of Oxford & University of Leipzig
14:50 – 15:15
G protein-mediated signal transduction of adhesion GPCR
Ines Liebscher, University of Leipzig
15:15 – 15:40
Real-time monitoring of GPCR signaling in living cells: from intracellular signaling microdomains to single molecules
Davide Calebiro, University of Würzburg
15:40 – 16:30
Coffee break
16:30 – 17:30
General Meeting of the Adhesion-GPCR Consortium
19:00
Evening program

Saturday, Sep 8 2012

Session G – Adhesion-GPCR in endocrine, cardiovascular, and immune functions

9:00 – 9:25
The origin of the Adhesion-GPCR family
Helgi Schiöth, University of Uppsala
9:25 – 9:50
The ADHD-susceptibility gene *lphn3.1* modulates dopaminergic neuron formation and locomotor activity during zebrafish development
Klaus-Peter Lesch, University of Würzburg
9:50 – 10:15
Adhesion 7TM receptors – major players in the endocrine and enteroendocrine system
Thue Schwartz, University of Copenhagen
10:15 – 10:45
Coffee break
10:45 – 11:45
Future initiatives of the Adhesion-GPCR community
11:45 – 12:00
Concluding remarks
Jörg Hamann, University of Amsterdam & Tobias Langenhan, University of Würzburg