

New functions and signaling mechanisms for the class of adhesion G protein–coupled receptors

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The class of adhesion G protein–coupled receptors (aGPCRs), with 33 human homologs, is the second largest family of GPCRs. In addition to a seven-transmembrane α -helix—a structural feature of all GPCRs—the class of aGPCRs is characterized by the presence of a large N-terminal extracellular region. In addition, all aGPCRs but one (GPR123) contain a GPCR autoproteolysis–inducing (GAIN) domain that mediates autoproteolytic cleavage at the GPCR autoproteolysis site motif to generate N- and a C-terminal fragments (NTF and CTF, respectively) during protein maturation. Subsequently, the NTF and CTF are associated noncovalently as a heterodimer at the plasma membrane. While the biological function of the GAIN domain–mediated autocleavage is not fully understood, mounting evidence suggests that the NTF and CTF possess distinct biological activities in addition to their function as a receptor unit. We

discuss recent advances in understanding the biological functions, signaling mechanisms, and disease associations of the aGPCRs.

Keywords: adhesion G protein–coupled receptor; signal transduction; structural biology; development; myelination; synaptogenesis; cancer

Introduction

Two years have passed since the 6th International Adhesion GPCR (G protein–coupled receptor) Workshop.¹ In that time, international collaborations have evolved, a new nomenclature has been established (www.adhesiongpcr.org), and significant advances in ligand identification and delineation of activation mechanisms for adhesion G protein–coupled receptors (aGPCRs) have been made. Most aGPCRs undergo a GPCR autoproteolysis–inducing (GAIN) domain–mediated autoproteolysis process at the GPCR autoproteolysis site (GPS) to generate N- and a C-terminal fragments (NTF and CTF, respectively; Fig. 1). NTF- and CTF-mediated domain-specific functions are another fast-developing area in the field of aGPCRs.

How aGPCRs are activated and which intracellular pathways respond to aGPCR activation signals have been a focus in the field for the past 2 years. Several groups in Europe and the United States have tackled these questions with a large array of assays, from classic *in vitro* approaches to complex *in vivo* analyses. Together, a picture of aGPCR signaling has emerged that includes two different models. In the first, referred to as *trans* and *cis* signaling, signals are transduced by both the NTF and CTF. In the second model, a self-activation scenario derived from receptor fragmentation is proposed. Although aGPCRs have been classified as being aGPCRs based on structural similarities, only a few of them have characterized downstream signaling pathways.

The 7th International Adhesion GPCR Workshop

The 7th International Adhesion GPCR Workshop was held at the Boston Children’s Hospital, Harvard Medical School, June 5–7, 2014 (Appendix S1), and included 70 scientists from 15 countries. It featured 32 oral presentations and 27 posters from a variety of research fields, including signal transduction, evolution, structural biology, developmental biology, neurobiology, human diseases, and immunology.

Evolution and structure of aGPCRs

Helgi Schiöth (Uppsala University). Helgi Schiöth presented evolutionary studies on the aGPCRs, showing that these receptors are of ancient origin and found in all vertebrates, as well as primitive animals and unicellular metazoans. Adhesion GPCRs with short extracellular regions are found in several basal fungi, indicating that the aGPCRs are likely to have evolved before the split of unikonts from the common ancestor of eukaryotes about 1275 million years ago.² Adhesion GPCRs are likely to be ancestral to the secretin GPCRs (class B), as secretin GPCRs probably diverged from a specific family of aGPCRs; they are also present in choanoflagellates (a group of free-living unicellular and colonial flagellate eukaryotes). These are likely to be ancestral versions of aGPCRs that evolved more specified functions over the course of metazoan multicellularity. Several gene-mining studies have also delineated the early evolution and diversification of extracellular domains; such examples would be the emergence of the characteristic aGPCR domains—GPS and calx- β in the unicellular filasterean *Capsaspora owczarzaki* and EGF-CA in free-living unicellular organisms such as the choanoflagellate *Salpingoeca rosetta*.

The aGPCRs were classified into nine distinct families (with VLGR1 as one family) in humans according to the molecular signature of their seven-transmembrane (7TM) regions and extracellular domains.³ From an evolutionary perspective, potential homologs for genes belonging to families I, III, IV, V, and VIII are present in most invertebrates, including ascidians, lancelets, acorn worms, and cnidarians, whereas the families II, VI, and VII are more likely to be vertebrate specific. The acorn worm (*Saccoglossus kowalevskii*) is a hemichordate belonging to the superphylum of deuterostome bilateral animals. This genome is rich in GPCRs, with at least 18 aGPCRs, and five of the eight main human aGPCR groups are represented.⁴ The hemichordate aGPCR repertoire has sequences with N-terminal domains that are not commonly found within this

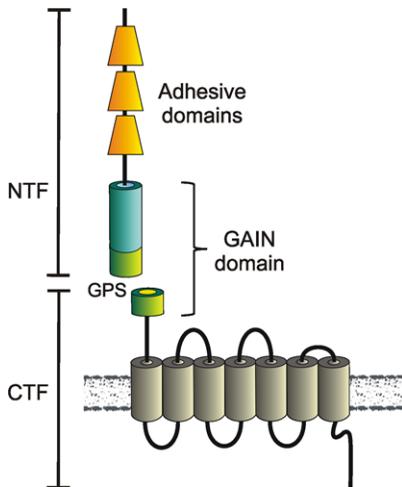


Figure 1. Structure topology of aGPCRs. Adhesion GPCRs are characterized by a large N-terminus that features several adhesive and functional domains. While the adhesive domains are thought to play roles in cell–cell or cell–matrix interactions, the highly conserved GPCR proteolysis site (GPS), is part of a bigger GPCR autoproteolysis-inducing (GAIN) domain. Most aGPCRs undergo a GAIN domain-mediated autoproteolysis process at the GPS during protein maturation to generate an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain in the form of a noncovalently associated heterodimer on the plasma membrane.

family. Especially interesting is the protein sequence with four hyalin repeats (HYR), von Willebrand factor (vWF) type D domain (VWD), and the astacin domains that are recognized to have cell adhesion properties. The results suggest that 14 of the 18 hemichordate aGPCRs have the GPS domain. The exceptions include one sequence that has four EGF-CA repeats, found usually in group II, another sequence that has TSP1 repeats that are found in group VII, and another that has a lectin C domain. It is noteworthy that about 80% of the human aGPCR N-terminal domains are explicitly found in the aGPCRs of the acorn worm. The aGPCRs are found in the earliest diverging phyletic branches of the metazoa, the sponges (*Amphimedon queenslandica*) and the comb jelly (sea walnut), a ctenophore (*Mnemiopsis leidyi*).⁵ The sponge genome encodes several genes for the aGPCRs, of which many lack the characteristic GPS motif, while, perhaps more surprisingly, 13 of those aGPCRs have a hormone-binding motif (HRM) domain in their N-termini, although no hormones have yet been reported in the sponge. This suggests that these primitive

metazoans have a rich repertoire of aGPCRs, as well as a wide range of N-terminal domains, similar to many vertebrates. Preliminary analysis suggests that the primitive comb jelly has several aGPCRs with N-terminal domains. Together, the studies described by Schiöth reveal that the aGPCRs have an ancient origin and are likely to be one of the early components allowing cells to adhere and communicate during the evolution of multicellularity.

Demet Araç (University of Chicago). Araç presented her group's recent progress since the determination of the first crystal structure of the GAIN domain of aGPCRs, a decisive step toward understanding the structure–function relationships of these giant receptors.⁶ Araç is interested in understanding the mechanism of aGPCR function using structural, biochemical, and functional methods. The GAIN domain is juxtaposed with the transmembrane domain and there is a short linker between the two domains. The remaining N-terminal extracellular domains vary between different aGPCRs and are likely important for each protein's specific function. Growing evidence suggests that the GAIN domain and the CTF act together to regulate receptor function. Autoproteolysis occurs between the last two β -strands of the GAIN domain. Upon autoproteolysis, the two pieces remain associated. It is possible that a large protein ligand may bind to the GAIN domain and remove the majority of the GAIN domain, leaving the last β -strand free for the activation of the receptor. Because of the hydrophobic nature of the remaining β -strand, it will immediately associate with the hydrophobic transmembrane helices of the receptor.

Araç and colleagues work on all three regions of aGPCRs: the GAIN domain, the transmembrane helices, and the other extracellular regions. To understand the function of the GAIN domain, they engineered protein binders for various GAIN domains using phage and yeast display technologies. These binders are potential ligands that may regulate receptor activity. They will investigate novel endogenous ligands for GAIN domains and study the function of the GAIN domain *in vivo* in animal systems. To understand the function of the transmembrane helices, the Araç lab will determine their three-dimensional structure using X-ray crystallography and visualize their solution structure using electron microscopy. To understand the

specific function of the other extracellular regions, Araç and colleagues will use different aGPCRs with known binding partners and determine their structures in complex with their binding partners. They will use the acquired structural and biophysical data to investigate the role of aGPCRs in functional assays.

Susanne Ressler (Stanford University). Ressler presented the first structural data on a ligand of a representative aGPCR, brain-specific angiogenesis inhibitor 3 (BAI3). At present, the only known ligands for BAI3 are C1Q-like (C1QL) proteins. The functions of C1QL proteins are not known, but they bind via their globular C1q (gC1q) domains in a Ca^{2+} -dependent fashion to the thrombospondin repeats of BAI3,⁷ an aGPCR that has been implicated in schizophrenia. Thus, C1QL proteins may have a function in synapse homeostasis by binding with high affinity to BAI3. C1QL1 is primarily expressed in the brain stem, C1QL2 in the hippocampus and thalamus, and C1QL3 in the thalamus, hippocampus, and neocortex;⁸ this may reflect distinct physiological roles in the brain. C1QL proteins are composed of an N-terminal signal peptide, followed by a sequence of 15 residues with two conserved cysteine residues important for higher-order oligomerization,⁸ a spacer region of 15–35 residues, 17 G-X-Y collagen-like repeats, and a C-terminal gC1q domain of approximately 140 residues. The presence of a gC1q domain is the hallmark of the C1q/TNF (tumor necrosis factor) superfamily, whose members are involved in various physiological functions including synapse homeostasis.^{7,9} Ressler *et al.* (in revision) recently solved high-resolution crystal structures of the globular C1q domains of C1QL1, C1QL2, and C1QL3, revealing ion-binding sites at two major regions of the gC1q domain trimer. Electrostatic potential maps of the C1QL structures show a conserved negatively charged pattern distinct from other C1q-domain proteins. Several structures of gC1q domains have been determined by X-ray crystallography; adding additional C1QL structures to the list of C1q/TNF superfamily members of known structure underlines the remarkable conservation of the gC1q domain across all members and its success in evolution. Cerebellin-1 (Cbln1), a member of the C1q/TNF superfamily for which no crystal structure has been solved to date, functions at the synapse.

Cbln1 is a secreted protein selectively expressed in the central nervous system (CNS) that binds simultaneously to the postsynaptically localized $\delta 2$ glutamate receptor (GluR $\delta 2$) and the presynaptically localized neurexin protein in a Ca^{2+} -independent fashion.^{9,10} The formation of this tripartite protein complex results in enhanced synapse formation in the cerebellum and is crucial for motor learning.^{9,10} C1QL proteins may have similar functions to Cbln1. However, C1QL and Cbln1 proteins slightly differ in their domain architecture. Cbln1 lacks the N-terminal collagen-like domain, and thus is apparently restricted in its ability to form larger oligomers. However, they share conserved amino acids at the gC1q domain that could be key players in receptor recognition. Ressler suggested that C1QL proteins may have at least two major distinct receptor-recognition sites and thus could interact with more than one receptor, potentially depending on their localization in the brain. It remains a matter of speculation whether binding of C1QL proteins to BAI3 results in the release of the NTF from the CTF or whether stabilization of the NTF to the CTF occurs, and what event triggers a signal cascade.

Signaling mechanisms of aGPCRs

Ines Liebscher (University of Leipzig). Liebscher presented evidence for an activation mechanism of aGPCRs through a potential tethered peptide agonist. Since the first functional proof of G protein coupling was demonstrated for one member of the aGPCR class, GPR133,^{11,12} other family members, including GPR56,^{13–15} GPR126,¹⁶ and GPR114,¹⁷ have been shown to signal via G proteins.^{12,18} Liebscher *et al.* have continued the survey of G protein-mediated signal transduction with the aim of characterizing the whole aGPCR class; they have analyzed 18 of the 33 members encoded in the human genome. Taking advantage of this broad range of aGPCRs, they aim to decipher common activating mechanisms based on sequence comparison and mutation analysis of highly conserved motifs. Previous studies showed that N-terminally truncated aGPCRs display constitutive activity,¹⁴ leading to two proposed activation scenarios: (1) the CTF is constrained by a tethered inverse agonist located in the N-terminus upstream of the GPS cleavage site such that deletion of the N-terminus releases this constraint, and (2) the N-terminus

downstream of the GPS cleavage site contains a tethered agonist, which is exposed by deletion of the N-terminus upstream of the GPS, thereby allowing its interaction with the CTF. Liebscher *et al.* showed that several N-terminally truncated aGPCRs display the expected constitutive activity, supporting both scenarios. Further mutational analysis, however, led to the conclusion that at least three aGPCRs are activated through a tethered peptide agonist, rather than being kept inactive through an inverse tethered agonist.

Randy Hall (Emory University School of Medicine). Hall presented studies on signal transduction of brain-specific angiogenesis inhibitor 1 (BAI1). BAI1–3 compose a subfamily of aGPCRs that are highly expressed in the brain and were first studied for their ability to inhibit angiogenesis and tumor formation.¹⁹ In recent work, Hall and colleagues found that overexpression of BAI1 results in activation of the Rho pathway via a $G\alpha_{(12/13)}$ -dependent mechanism, with truncation of the BAI1 N-terminus associated with a dramatic enhancement in receptor signaling.²⁰ These results are similar to previous findings for other aGPCRs.²¹ The constitutive activity of the truncated BAI1 mutant also resulted in enhanced downstream phosphorylation of ERK, as well as increased receptor association with β -arrestin-2 and increased ubiquitination of BAI1. To gain insight into the regulation of BAI1 signaling, Hall's laboratory screened the C-terminus of BAI1 against a proteomic array of PDZ domains to identify novel interacting partners. The screens revealed that the BAI1 C-terminus interacts with a variety of PDZ domains from synaptic proteins. Biochemical fractionation studies further showed that BAI1 is highly enriched in postsynaptic density fractions, a finding consistent with the observation that BAI1 can interact with proteins known to be concentrated in the postsynaptic density and with recent independent findings from Tolia and colleagues.²² Studies are currently in progress to assess potential neuroanatomical, neurophysiological, and cognitive changes in mice lacking BAI1, in order to assess whether BAI1 might have an important role in synaptic function *in vivo*, in addition to the role in muscle development described by Ravichandran and colleagues.²³ Hall's recent findings demonstrate that BAI1 is a synaptic receptor that can activate both the Rho and ERK pathways, with the NTF and CTF

playing key roles in the regulation of BAI1 signaling activity.

Gregory G. Tall (University of Rochester Medical Center). Tall described work in progress designed to decipher the signaling mechanisms of GPR56 and GPR110. They chose to investigate G protein coupling of a panel of aGPCRs, with an emphasis on using GPR56 and GPR110 for detailed mechanistic studies. GPR56 has two putative ECM ligands^{13,24} and a $G_{12/13}$ -dependent signaling mechanism;^{13–15} it regulates cortical neuron migration during brain development and may have pathophysiological roles in cancer progression.^{13,24} GPR110 is an orphan receptor about which little is known, other than that its expression is upregulated in lung adenocarcinomas and prostate cancers.²⁵ To address the signaling mechanism(s) of these receptors, Tall and colleagues developed a new technology using the G protein α subunit molecular chaperones, Ric-8A and Ric-8B, to purify milligram quantities of all four classes of $G\alpha$ subunits.^{26–28} G protein heterotrimers were assembled from these purified, recombinant $G\alpha$ subunits ($G\alpha_{i1}$, $G\alpha_o$, $G\alpha_q$, $G\alpha_{13}$, $G\alpha_s$) and purified $G\beta_1\gamma_2$ heterodimer. The heterotrimers ($G_{i/o}$, G_q , G_{13} , G_s) were precoupled to membranes prepared from aGPCR-expressing High-Five or *Sf9* insect cells. The aGPCR-influenced [³⁵S]-GTP γ S binding kinetics of each G protein heterotrimer subtype was measured using an established nitrocellulose filter-binding assay. These measurements allow direct determination of the G protein-coupling profiles of particular aGPCRs. Tall and colleagues denatured and/or extracted the NTFs from the membrane preparations using urea treatment, which permits measurement of the activity of the CTFs in the absence of NTF inhibition. Their preliminary results support two mutually exclusive hypotheses: (1) aGPCRs are constitutively inhibited receptors in which NTFs act as natural inverse agonists to the CTFs; or (2) removal/rearrangement of the NTF reveals a tethered agonist element that stabilizes an active CTF conformation. Additional experiments are being planned on recombinant full-length and truncated NTFs on CTF, and on the influence of purified TG2, collagen III, and/or extracellular matrix on full-length receptor activity. Identifying the G protein-coupling specificities of aGPCRs and demonstrating NTF inhibition of the respective CTF suggests a potential common mechanism of

action that may be applicable to the entire aGPCR class.

Miriam Peeters (University of Copenhagen). Peeters presented work from the group of Thue Schwartz (University of Copenhagen) on the signaling of aGPCRs. At the 6th International Adhesion GPCR Workshop, Schwartz proposed an activation mechanism for aGPCRs in which (part of) the NTF acts as a tethered inverse agonist that silences the constitutively active CTF.¹ However, at that time, the signaling pathways involved were rather unclear for most aGPCRs. Moreover, it was unknown which and how many intracellular signaling pathways could be effected in the absence of the N-terminus. Peeters presented work on GPR112, one of the largest aGPCRs, with more than 3000 amino acids and a mass of approximately 500 kDa.²⁹ Full-length and N-terminally truncated constructs of GPR112 were studied *in vitro* in a variety of *in vitro* signaling models and using a number of pathway inhibitors, including selective G protein inhibitors, such as pertussis toxin, and second-messenger inhibitors, such as the Rho kinase inhibitor Y27632. The full-length receptor was found to have constitutive activity via several G protein pathways (this is consistent with what is known about the extensively studied class A GPCRs, which activate multiple pathways via G protein-dependent and -independent effectors,³⁰ and the observation that many GPCRs are partially constitutive active³¹). A truncated construct of GPR112 lacking the complete N-terminus upstream of the autoproteolytic cleavage site demonstrated an increase in constitutive activity through pathways observed for the full-length receptor, similar to what has been described for GPR56, where an increased level of activated RhoA was seen only when the CTF was expressed in HEK293 cells.³¹ In the GPR112 experiments described by Peeters, the CTF was able to couple with additional G proteins, and a large increase in G protein-independent signaling was observed. All signaling pathways tested appeared to be involved in the constitutive signals seen in the N-terminally truncated GPR112.

Domain-specific functions and in vivo signaling of aGPCRs

Simone Prömel (University of Leipzig). Prömel presented her group's work on analyzing signal transduction of the invertebrate latrophilin *in vitro* and *in vivo*. Latrophilins (LPHN, CIRL/CL) were

first studied owing to their ability to bind α -latrotoxin, a component of the black widow spider toxin. Interaction with α -latrotoxin leads to calcium-independent exocytosis of neurotransmitters in various neurons.^{32,33} Latrophilins comprise one of the oldest subclasses of aGPCRs. In the model organism *Caenorhabditis elegans*, Prömel and colleagues previously showed that the latrophilin homolog lat-1 is involved in developmental processes and in fertility.^{34,35} However, the molecular mechanisms of receptor activation and the signals through which each lat-1 mode of function is brought about are largely unknown. Importantly, while one of the three mammalian latrophilin homologs has been shown to couple to a $G\alpha_o$ subunit,³⁶ extensive analysis for other latrophilins has not been done. Prömel's group has used *in vivo* and *in vitro* approaches to clarify the signaling of lat-1 in *C. elegans*, focusing on the CTF-dependent mode of lat-1. Their data provide strong evidence that lat-1 couples to G proteins. To assess physiological relevance, they used an *in vivo* assay based on rescue of cell division-plane orientation and spindle-polarity defects *lat-1* mutant embryos. Prömel discussed potential novel insights into latrophilin function on a molecular level to specifically link one of its biological functions with a signaling cascade on a cellular level.

Felix Engel (University of Erlangen-Nürnberg).

Engel and colleagues have been studying the function of Gpr126 during heart development. Like other aGPCRs, Gpr126 has a CTF and an NTF, which are believed to stay noncovalently associated after cleavage.³⁷ A physiological function for Gpr126 was first described in the zebrafish model. Analysis of *gpr126^{st49}* mutant zebrafish showed that Gpr126 is required for the initiation of myelination by Schwann cells.³⁸ In contrast, *Gpr126* deletion in one mouse model was shown to be embryonically lethal before myelination occurs;³⁹ in another *Gpr126* mutant mouse model with a less penetrant embryonic lethal phenotype, and in a conditional knockout mouse model, Gpr126 was found to be required for myelination in mice.^{16,40} A detailed analysis of the *gpr126^{st49}* mutant fish showed neither a heart phenotype nor embryonic lethality.⁴¹ As the *st49* mutation introduces a stop codon just before the GPS domain, the *gpr126^{st49}* mutant fish might express a functional NTF that functions independently of its CTF, and thus the NTF,

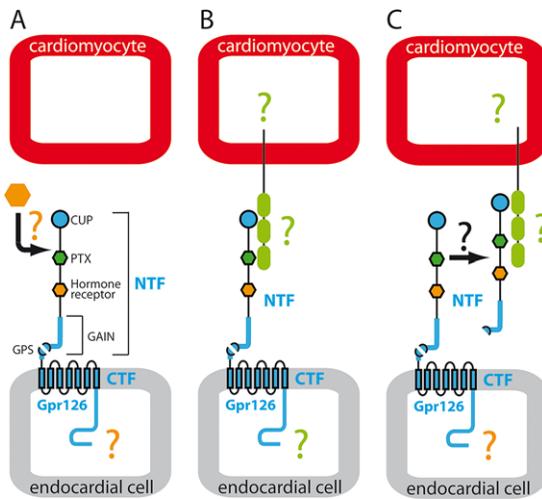


Figure 2. Possible GPR126 signaling modalities during heart development. GPR126 possesses a GPS motif and an extended NTF containing a CUB (Complement, Uegf, Bmp1) domain, a PTX (pentraxin) domain, and a hormone-binding domain. Study results in Engel's lab indicate that GPR126 is expressed in endocardial cells (ECs) during mouse heart development. Since ECs and cardiomyocytes exhibit cellular defects in *Gpr126* knockout mice, Engel and colleagues hypothesize that EC function depends on CTF-mediated signaling, while cardiomyocyte function depends on NTF-mediated signaling. (A) Signaling in ECs depends on ligand binding to the NTF, inducing CTF-dependent signaling. (B) Interaction of the NTF with a receptor or cell surface molecule induces CTF-dependent signaling in ECs and an unknown signaling pathway in cardiomyocytes. (C) Cleavage and dissociation of the NTF induces CTF-dependent signaling in ECs. The shed NTF induces signaling in cardiomyocytes.

but not the CTF, may be required for heart development. A detailed analysis of three *Gpr126* knockout mouse models and four *gpr126* zebrafish morphants revealed several heart phenotypes.⁴¹ The mitochondrial and the trabeculation phenotypes were also observed in *gpr126* zebrafish morphants. Experiments demonstrated that the NTF fragment NTF^{ΔGPS} (amino acid 1–783) was sufficient to rescue the cardiac trabeculation phenotype, but not the myelin gene expression phenotype,⁴¹ confirming the hypothesis that the NTF, but not the CTF, is required for the developing heart. *In situ* hybridization expression analysis indicated that, at least at E11.25, *Gpr126* expression is restricted to endocardial cells (ECs). Therefore, Engel proposed three possible Gpr126 signaling modalities during heart development (Fig. 2): (1) signaling in ECs depends on ligand binding to the NTF, which induces CTF-

dependent signaling; (2) interaction of the NTF with a receptor or cell surface molecule induces CTF-dependent signaling in ECs and an unknown signaling pathway in cardiomyocytes; and (3) cleavage and dissociation of the NTF induces CTF-dependent signaling in ECs. In this model, the shed NTF also induces signaling in cardiomyocytes. To determine if the heart phenotype is due to defects in endocardial Gpr126, they generated conditional *Gpr126* mice. These mice can produce reporter knockouts, conditional knockouts, and potentially null alleles (disrupting *Gpr126* after exon 6 early in the NTF), following exposure to the site-specific recombinases Cre and Flp; in addition, *Gpr126* expression patterns, based on a *lacZ* expression cassette, can be determined. The Engel lab has also initiated experiments to identify possible interaction partners on the cardiomyocyte plasma membrane, partners that mediate NTF-dependent signaling in this cell type.

Adhesion GPCRs in organ development

Mounting evidence suggests that aGPCRs regulate organogenesis. The 7th International Adhesion GPCR Workshop featured the presentation of two major works on GPR116 in lung development, as well as an unpublished report on GPR56 involvement in the regulation of muscle hypertrophy.

James Bridges (Cincinnati Children's Hospital Medical Center). Bridges reported on Gpr116 and its role in pulmonary surfactant production. *Gpr116* mRNA expression is highly enriched in the adult mouse lung and is detected at lower levels in other organs, including the heart, kidney, stomach, and adipose tissue. In the lung, GPR116 is expressed on the plasma membrane of alveolar type I and II cells; data from Bridges' lab, as well as two independent labs, have demonstrated that *Gpr116*-deficient mice show a progressive, postnatal accumulation of alveolar surfactant (Fig. 3).^{42–44} The data demonstrate that epithelial GPR116 is essential for surfactant homeostasis and implicate this receptor as a potential drug target for modulating alveolar surfactant levels in the context of lung disease. Recent unpublished data from Bridges' lab demonstrate that *Gpr116*-deficient primary type II cells have increased basal and stimulus-induced surfactant secretion rates, associated with increased ATP- and thapsigargin-induced calcium responses, indicating that intra- and extracellular calcium handling

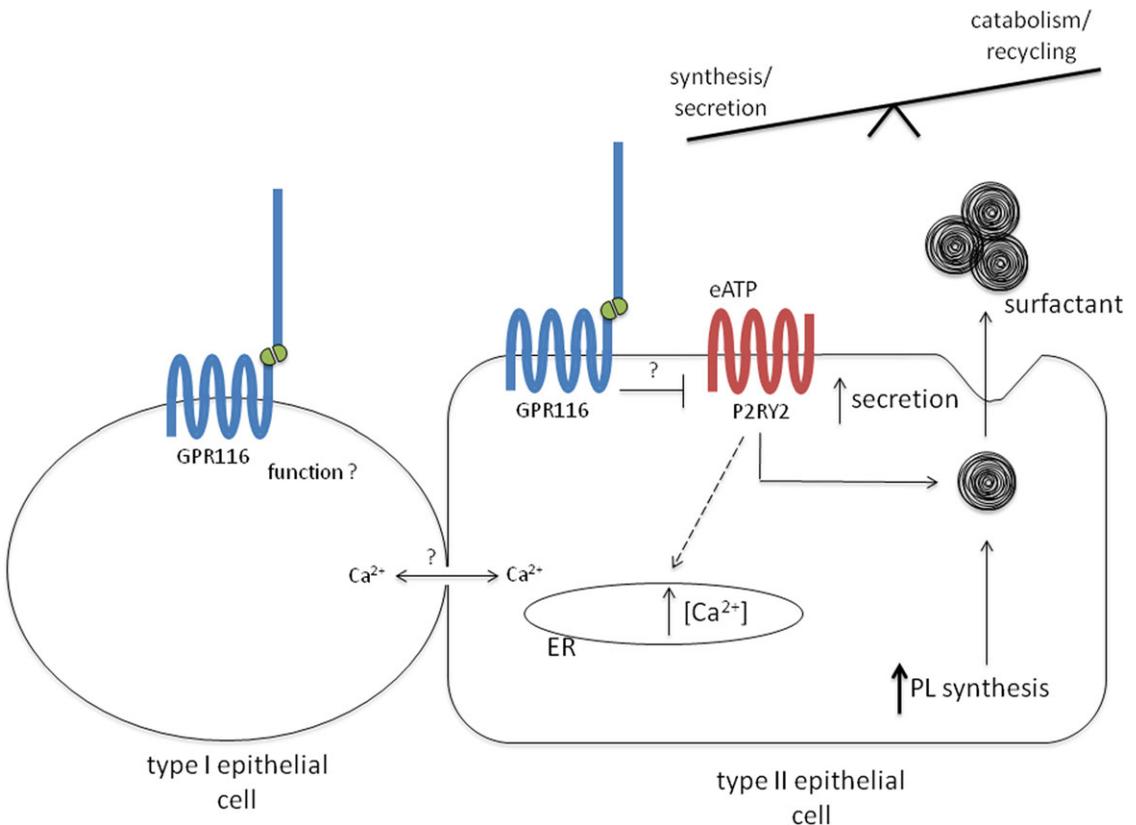


Figure 3. Working model for GPR116 function in pulmonary surfactant homeostasis. Loss of GPR116 function in mice perturbs the balance of surfactant synthesis and secretion versus catabolism and recycling, resulting in surfactant accumulation in the airspaces. Surfactant accumulation in *Gpr116* knockout animals is associated with increased phospholipid (PL) synthesis and secretion, and increased ATP- and thapsigargin-induced calcium flux, in type II epithelial cells. ATP/P2RY2 signaling is known to stimulate surfactant secretion *in vitro*, suggesting that GPR116 may modulate P2RY2 activity, or other receptor-dependent pathways implicated in PL secretion such as ADRB2 or ADORA2b, to control surfactant homeostasis *in vivo*. GPR116 expression is also detected in type I alveolar epithelial cells; the specific function of GPR116 in this cell type has yet to be determined.

is altered in the absence of GPR116; current studies are underway to determine the underlying mechanism. To identify ligands and potential interacting partners of GPR116, they have used pull-down/mass spectrometry approaches with full-length GPR116 and truncation mutants that demonstrate constitutive G protein–coupled signaling activity. Through these approaches, Bridges and colleagues have identified putative interacting proteins that bind to the inactive and active forms of GPR116; work in progress is aimed at determining the importance of these protein–protein interactions in the context of calcium handling and surfactant homeostasis.

Donna Mareta Ariestanti (Tokyo Institute of Technology). Ariestanti described how *Gpr116*

deficiency in mice is associated with features of chronic pulmonary disease. Alveolar macrophages (AMs) are phagocytic for pathogens and are important in both innate and acquired immunity in the respiratory tract. A marked increase of AMs has been seen in patients with emphysema, which correlates AMs with the pathophysiology of emphysema. *Gpr116* possesses long immunoglobulin (Ig)-like repeats in the extracellular region and is expressed in alveolar type II cells. Previously, mice lacking *Gpr116* were shown to exhibit significant accumulation of lung surfactant, indicating an essential role for *Gpr116* in surfactant homeostasis. *Gpr116*^{−/−} mice also exhibit emphysema-like symptoms, with enlarged alveoli, accumulation of foamy AMs, and increased expression of MMP-12.⁴³ Ariestanti and

colleagues hypothesize, therefore, that Gpr116 may be involved in the pathogenesis of emphysema. They are now investigating whether emphysema development in *Gpr116*^{-/-} mice is caused by ROS accumulation and NF- κ B activation in AMs.

James P. White (Dana-Farber Cancer Institute).

A postdoctoral fellow in Bruce M. Spiegelman's laboratory, White presented studies on the role of GPR56 in the regulation of muscle hypertrophy. Spiegelman's lab previously showed that a splice isoform of PGC-1 α , PGC-1 α 4, induces muscle hypertrophy through the induction of IGF-1 and the suppression of myostatin.⁴⁵ The discovery of PGC-1 α 4 has provided an interesting tool for identifying new mediators of muscle hypertrophy.⁴⁵ Unlike PGC1 α 1, PGC1 α 4 does not appear to regulate a broad program of mitochondrial gene expression; rather, its function seems to be focused on muscle hypertrophy and strength. White presented work showing that GPR56 and its ligand collagen III are transcriptional targets of PGC-1 α 4 in muscle cells. Forced expression of *Gpr56* in myotubes resulted in myotube hypertrophy, which was strongly dependent on G α _{12/13} signaling. Others have shown that GPR56 loss of function does not affect myotube

size, at least in the unprovoked state.⁴⁶ Together, these data support the conclusion that GPR56 signaling, while part of the stimulus-provoked hypertrophic process, has a minimal role in basal early-stage myogenesis or myotube maturation. Signaling through G α _{12/13}/Rho has been associated with muscle hypertrophy in pressure-overloaded cardiac muscle,⁴⁷ supporting the concept of G α _{12/13} being a mechanosensitive anabolic pathway.

White described work showing that *GPR56* mRNA expression is induced during models of muscle hypertrophy in both human and mouse; this is in contrast to their observations with endurance-based exercise, where no change in *GPR56* expression is observed. These results are consistent with GPR56 elevations being selective for conditions of muscle hypertrophy, including mTOR activation (Fig. 4). In addition, *Gpr56* expression appeared to be very important in mediating at least some of the hypertrophic response of mechanical overload in mice. The translational impact of these finding implies that variations in *Gpr56* expression due to disease or aging could affect the ability of muscle to adapt to mechanical loading. The ability of skeletal muscle to adapt to mechanical stimuli is essential for

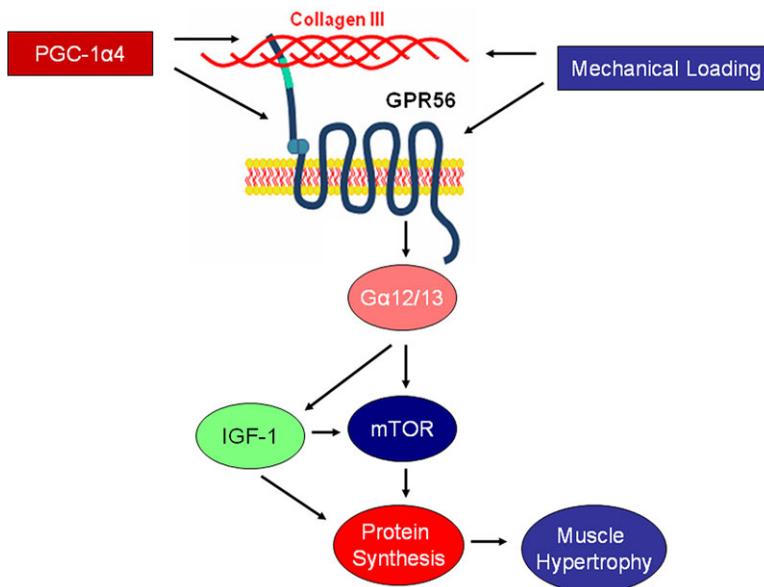


Figure 4. Working model for GPR56 signaling in skeletal muscle. In response to mechanical overload, GPR56 signals through the G α _{12/13} subunit to activate mTOR and downstream protein synthesis. GPR56 gain of function induces IGF-1 mRNA expression, which is dependent on a functional G α _{12/13} subunit. Together, GPR56 signaling is a novel pathway linking mechanical loading to muscle anabolism.

health and well-being, especially during conditions of sarcopenia or cachexia where muscle mass is compromised. The GPR56 signaling pathway is ripe for further scientific exploration, including the potential pharmaceutical interventions against muscle atrophy.

Adhesion GPCRs in the nervous system: myelination

The involvement of aGPCRs in the nervous system has been a hallmark of the field of aGPCRs for many years. Several groups presented new insight into the functions of aGPCRs in myelination, neural development, and synaptogenesis.

Stefanie Giera (Harvard Medical School). A postdoctoral fellow from Xianhua Piao's lab (Harvard Medical School), Giera presented unpublished work on GPR56 and CNS myelination. Myelin, the multilayered membrane synthesized by glial cells (oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS)), provides insulation and trophic support to axons. In addition to the malformed cerebral cortex, magnetic resonance imaging (MRI) of bilateral frontoparietal polymicrogyria (BFPP) brains also shows myelination defects in the white matter (Fig. 5),^{48,49} suggesting a role for GPR56 in CNS myelination. Giera and colleagues demonstrated that GPR56 is present in oligodendrocyte precursor cells and immature oligodendrocytes, consistent with a previous report in rats.⁵⁰ Further-

more, Giera presented data from mouse models and tissue culture that support a role for Gpr56 in oligodendrocyte precursor cell development.

Rong Luo (Harvard Medical School). Also from Xianhua Piao's group, Luo presented unpublished studies on the search for GPR126 ligands in Schwann cell development and PNS myelination. In addition to the GAIN domain-mediated autoproteolysis process, mammalian GPR126 has an S2 site (amino acid 441 in mice) where furin-mediated cleavage occurs.⁵¹ GPR126 is essential for PNS myelination and heart development.^{16,38,40,52} However, the ligand (or ligands) of GPR126 during Schwann cell and cardiac development is unknown. To identify the ligand of GPR126, Luo and colleagues generated three human IgG Fc-tagged mouse GPR126 fusion proteins that contain either the full-length NTF (GPR126N31–807) or fragments N-terminal (GPR126N31–438) or C-terminal (GPR126N446–807) to the S2 site. Ligand binding *in situ* revealed the ligand-expressing cell types in the early postnatal PNS and heart. Preliminary analysis of candidate ligands suggests that GPR126 has distinct binding partners in peripheral nerves compared to the heart.

Amit Mogha (Washington University School of Medicine). A postdoctoral fellow from Kelly Monk's group (Washington University School of Medicine), Mogha discussed the function of GPR126 in myelin maintenance and remyelination. Previous reports demonstrated that GPR126 is essential for Schwann cell development and the initiation of myelination in the zebrafish and mouse PNS.^{38,40} More recently, Mogha *et al.* showed that GPR126 performs these functions by directly regulating cAMP concentrations via coupling to heterotrimeric G proteins in Schwann cells.¹⁶ Interestingly, the expression of *Gpr126* is maintained in adult Schwann cells, suggestive of a function in the adult PNS. Mogha *et al.* have therefore begun to analyze the role of Gpr126 in myelin maintenance and/or remyelination after injury by studying a Schwann cell-specific tamoxifen-inducible knockout *PlpCre-ERT2;Gpr126^{fl/fl}* mouse model. Preliminary data indicate that deletion of *Gpr126* in mature Schwann cells does not affect myelin maintenance, consistent with a recent report in zebrafish.⁵² Their aim is to define the role of GPR126 in demyelination and remyelination in order to better understand if

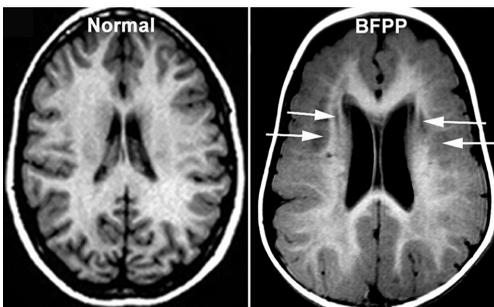


Figure 5. Myelination defects in BFPP brain. Loss-of-function mutations in *GPR56* cause a devastating human brain malformation called bilateral frontoparietal polymicrogyria (BFPP), in which the normal convoluted brain surface is replaced by numerous small gyri. In addition to this cortical defect, BFPP brains also show signs of myelination abnormalities. In contrast to the white matter in the normal brain (left), the BFPP brain (right) presents with signal changes (arrows) on MRI, indicating defective myelination. This figure is adapted from figure 1a in Ref. 49.

developmental signaling pathways are reinitiated in the regenerating nerve.

William Talbot (Stanford University). Talbot discussed his lab's studies on the role of GPR126 in myelination using genetic approaches to discover genes that are essential for the development of myelinated axons. A zebrafish genetic screen for mutants with abnormalities in myelinated axons identified two different mutations in *gpr126*.^{38,53} The initial characterization of markers indicated that although axons and Schwann cells are present in peripheral nerves of the mutants, the Schwann cells do not express *krox20*, a marker of Schwann cells that have commenced myelination.⁵³ More detailed studies and ultrastructural analysis showed that the Schwann cells do not progress beyond the promyelinating stage in *gpr126* mutants, indicating that this aGPCR is required for Schwann cells to initiate myelination.³⁸ Analysis of a mouse mutation indicated that *Gpr126* has a conserved function in myelination in mammals and defined additional roles for *Gpr126* in nerve development.⁴⁰ Analysis of chimeric embryos showed that wild-type Schwann cells can myelinate *gpr126* mutant axons, indicating that *Gpr126* acts autonomously to initiate myelination in Schwann cells.³⁸ Treatment with forskolin, which activates adenylyl cyclase to increase cAMP, restored myelin in *gpr126* mutants, providing evidence that *Gpr126* signals through a cAMP second messenger.^{38,52} Compact myelin was evident in *gpr126* mutant nerves weeks after a transient application of forskolin, indicating that *Gpr126* signaling is required for the initiation of myelination but not for the maturation or maintenance of myelin in Schwann cells.⁵² Expression of a constitutively active form of protein kinase A (PKA) restored myelin basic protein expression in nerves of *gpr126* mutants;⁵² this suggests that PKA acts downstream of *Gpr126*, as also reported in an independent study.¹⁶ An important outstanding question is the nature of the signals that activate *Gpr126*. Paavola *et al.* recently reported that type IV collagen binds to the N-terminal fragment of *Gpr126* and increases cAMP levels in HEK cells expressing *Gpr126*.⁵⁴ In addition, a truncated derivative of *Gpr126* lacking the N-terminal fragment is constitutively active,⁵⁴ suggesting that this region of the receptor antagonizes signaling in a way that is relieved by ligand binding, as previously suggested for other receptors.²¹

Kelly Monk (Washington University School of Medicine). Monk presented her lab's work on GPR126 in Schwann cell development. Schwann cells must radially sort axons into a 1:1 relationship before iteratively wrapping an axonal segment to form the myelin sheath. As noted above, PNS myelination requires GPR126,^{16,38,40,52} which has been proposed to have NTF- and CTF-distinct functions in heart development.⁴¹ Notably, mutations in *Lama2* phenocopy Schwann cell defects in *Gpr126* mutants. The Monk lab used mutant analysis in zebrafish and mice to dissect GPR126-NTF-mediated versus GPR126-CTF-mediated functions in Schwann cell development and myelination. Genetic and functional interactions between Laminin-211 and GPR126 were also discussed.

Adhesion GPCRs in the nervous system: neural development

Nicole Hartmann (University of Würzburg). From the laboratories of Tobias Langenhan and Robert Kittel (University of Würzburg), Hartmann described studies investigating aGPCR function using latrophilin/CIRL (calcium-independent receptor of latrotoxin). Several biochemical approaches have revealed putative latrophilin ligands,^{55,56} setting the stage for further insights into the signaling properties and physiological roles of latrophilin. Hartmann *et al.* have been using *Drosophila melanogaster* as a model organism to study latrophilin function *in vivo*. *Drosophila* contains only a single latrophilin homolog, which is one of only two aGPCRs present in the fly.

Previously, several studies documented different and seemingly unrelated roles for latrophilin in very different biological contexts, including synaptogenesis, planar cell polarity (PCP), and fertility.^{34,35,55} Furthermore, latrophilin possesses the capability to bind the α component of latrotoxin (α -LTX),⁵⁷ a potent neurotoxin secreted by the black widow spider, which induces massive release of neurotransmitter from synaptic terminals.⁵⁸ As a result of these findings, latrophilin was believed to play a direct role in synaptic transmission. However, Hartmann discussed preliminary data suggesting that loss of latrophilin leads to phenotypes very different from what would have been predicted based on these previous notions. These data unexpectedly link latrophilin function to mechanoreceptivity in sensory neuron populations (Gehring J., N. Hartmann

et al., submitted). A genomic-engineering strategy was used to generate *latrophilin* knockout and knock-in alleles. Surveillance of *latrophilin* gene activity revealed broad expression of the aGPCR in the CNS and PNS, including stretch- and touch-sensitive nerve cells. Accordingly, removal of *latrophilin* led to defective larval locomotion and reduced sensitivity to gentle touch; notably, basal synaptic transmission at the neuromuscular junction was largely unaffected. The presented findings indicate that mechanical stimulation is a physiological modality that can regulate the activity and thereby the signaling of aGPCRs.

André Goffinet (Université Catholique de Louvain). Goffinet presented his group's recent advances in deciphering the roles of two aGPCRs in neuronal development. CELSR3 and FZD3, members of the core PCP protein family, were previously shown to control forebrain axon guidance and wiring by acting in axons and/or guidepost cells.^{59,60} Whether they act in collaboration with or parallel to other axon-guidance cues is unknown, as are their roles in the PNS. Recent work from Goffinet's laboratory showed that CELSR2 acts redundantly with CELSR3 and that their combined mutation fully mimics that of *Fzd3*.⁶¹ Unexpectedly, forebrain wiring was normal in mice defective in *Vangl1* and *Vangl2*, showing that, contrary to epithelial PCP, axonal navigation is largely VANGL-independent. The phenotypes generated upon inactivation of *Fzd3* in different forebrain compartments were similar to those in conditional *Celsr2,3* mutants, indicating that FZD3 and CELSR2,3 function in *cis* in the same cells. Inactivation of either *Celsr2,3* or *Fzd3* in the dorsal thalamus does not affect forebrain wiring. In accordance with the handshake model, although joint inactivation in the cortex and thalamus adds little to cortical inactivation alone in terms of thalamocortical projections, it strongly perturbs the formation of the barrel field, indicating a role for handshake in cortical arealization.⁶¹ Goffinet and colleagues have also shown that CELSR3 and FZD3 play key roles in pathfinding of motor axons innervating the hindlimb.⁶² *Celsr3*- and *Fzd3*-deficient motor axons of the peroneal nerve segregate from those of the tibial nerve, but fail to extend dorsally and stall shortly after the branching point. Mutant axons respond normally to repulsive ephrinA:EphA forward signaling and glial cell-derived

neurotrophic factor. In contrast, they are insensitive to attractive EphA:ephrinA reverse signaling. In transfected cells, CELSR3 co-immunoprecipitates with ephrinA2/A5, RET, GFR α , and FZD3, indicating possible physical interactions. Like in the forebrain, the function of CELSR3 and FZD3 in motor axon guidance is *Vangl2* independent.⁶²

These genetic studies show that *Celsr2,3* and *Fzd3* regulate axonal navigation in the forebrain and in the limbs by using mechanisms different than classical epithelial PCP, and require interacting partners other than *Vangl1,2*, which remain to be identified. In motor neurons, CELSR3 and FZD3 interact with EphA:ephrinA reverse signaling to steer their axons in the hindlimb. Interactions with other guidance system are likely to occur and remain to be identified.

Adhesion GPCRs in the nervous system: synaptogenesis

Brian Ackley (University of Kansas). Ackley presented his lab's studies on the role of aGPCRs in synaptogenesis in *C. elegans*. The *C. elegans* genome encodes three conserved aGPCRs; two latrophilins/CIRL-like proteins, LAT-1 and LAT-2; and one Flamingo/CELSR-like protein, Fmi-1.^{34,63} Mutations in the *fmi-1* gene have been identified in a variety of genetic screens for defects in neural development, including axon pathfinding and synaptogenesis.^{63,64} Ackley *et al.* have extensively characterized the defects that occur in the ventral D-type (VD) neurons of *fmi-1* animals.⁶⁵ They have found that, in *fmi-1* mutants, VD neurons exhibit a low penetrance set of neurite-guidance defects; most interestingly, about 2% of the cells extend neurites toward the posterior, compared to wild-type animals, where 100% of the animals have anteriorly directed neurites. This phenotype is synergistically enhanced when animals are double mutant for mutations in *fmi-1* and various Wnt signaling components, including Wnt ligands (*lin-44*, *egl-20*), Frizzled receptor (*lin-17*), and disheveled proteins (*dsh-1*, *mig-5*).⁶⁵ Ackley and colleagues (unpublished data) have found that mutations in conserved Notch signaling genes can also synergize with *fmi-1* and with Wnt pathway mutations, suggesting that at least three different genetic pathways contribute to the fidelity of anterior neurite extension.

The VD neurons also exhibit a highly penetrant synaptic-patterning defect. Presynaptic markers

display a disorganized appearance and ultrastructure, and have an aberrant accumulation of electron-dense material.⁶⁴ Ackley and colleagues have found that similar defects are present in animals with mutations in *cdh-4*, a fat-like cadherin, and *let-502* Rho kinase, using presynaptic markers. Double mutants of either *cdh-4* or *let-502* and *fmi-1* are indistinguishable from *fmi-1* alone, suggesting that these genes function in the same genetic pathway as *fmi-1*. Also, mutations in *cdh-4* or *let-502* neither cause posterior axon outgrowth nor cause any increase in the penetrance of those defects in *fmi-1* mutant animals. These data indicate that the genetic origins of the posterior neurite extension and the synaptic patterning defects are likely distinct. Although FMI-1 is expressed in the nervous system, Ackley and colleagues find no evidence that FMI-1 is expressed in the VD neurons. They were able to rescue the posterior neurite and synapse formation defects completely when FMI-1 was expressed using the endogenous promoter; they were also able to partially rescue the synaptogenesis defects, but not the directional neurite-growth phenotype, when expressing FMI-1 specifically in the cholinergic motor neurons that are adjacent to the VD neurons.⁶⁴ Together, these data indicate that FMI-1 is functioning cell-non-autonomously, and confirm the independence of the two phenotypes.

Joseph Duman (Baylor College of Medicine).

From the lab of Kimberley Tolias (Baylor College of Medicine), Duman reported recent results on BAI1 and its involvement in dendritic spine formation. Duman *et al.* have reported that BAI1 plays an important role in excitatory-synapse development.²² BAI1 localizes to spines; loss of *BAI1* alters synaptogenesis and synaptogenesis in hippocampal neurons in culture and in somatosensory and cingulate cortex *in vivo*. The phenotype includes decreases in spine and synapse density and synaptic activity and persistence of immature spine morphology. These defects are rescued by full-length BAI1 in hippocampal neurons. BAI1 interacts with PAR3 and TIAM1, components of the evolutionarily conserved PAR polarity complex⁶⁶ required for normal synaptic development.^{67,68} Loss of BAI1 causes mislocalization of both proteins in neurons; they shift from dendrites to cell bodies. TIAM1 is a guanine nucleotide exchange factor (GEF), or activator, of RAC1, a small GTPase that promotes spine and

synapse formation. In *BAI1* knockdown neurons, RAC1 activation is decreased in spines and increased in dendritic shafts relative to control neurons. A BAI1 mutant protein that lacks its C-terminal TEV motif (BAI1 Δ TEV) does not interact with TIAM1/PAR3 and fails to rescue the spine, synapse, and PAR3 localization defects in BAI1 knockdown neurons. However, a BAI1 mutant protein that does not interact with the DOCK180/ELMO1 RAC1 GEF module required for BAI1-mediated phagocytosis in immune cells⁶⁹ rescues the spine phenotype as well as the wild-type protein. TIAM1 overexpression also rescues BAI1 knockdown spine phenotypes. Thus, BAI1 mediates spine and synapse development through PAR3/TIAM1/RAC1. Loss of BAI1 also affects the dendritic arbors of hippocampal neurons. BAI1 knockdown neurons have longer dendritic arbors and more dendritic tips at maturity than control neurons. These phenotypes are rescued by reintroduction of wild-type BAI1. Duman *et al.* modeled dendrite arborization in HEK293T cells, which exhibit an array of projections when expressing BAI1. These projections are highly polarized, largely emanating from a single pole. However, when BAI1 Δ TEV is expressed in these cells, the resulting arrays are decidedly unpolarized, and projections emanate from the entire cell periphery. Projection arrays caused by wild-type BAI1 are similarly unpolarized in the presence of dominant-negative CDC42. CDC42 is a small GTPase related to RAC1 that frequently functions upstream of the PAR complex.⁶⁶ These data further support the idea that BAI1 functions with the PAR complex to determine the striking polarity of the projection arrays in these cells and may function similarly in neurons.

S  verine M. Sigoillot (Coll  ge de France). A post-doctoral fellow in the lab of Fekrije Selimi (Coll  ge de France), Sigoillot presented work on BAI3 in neuronal development. BAI proteins are only present in vertebrates and are extremely well conserved (79% identity between human and zebrafish), and therefore could potentially control the development of complex cognitive abilities specific to vertebrates. The BAI proteins appear to contribute to defective behaviors in psychiatric diseases, as single nucleotide polymorphisms or copy number variants in the *BAI3* gene have been found to be associated with disorganized symptoms in schizophrenia and addiction,^{70,71} while loss of function of the *Bai2*

gene causes an antidepressant phenotype in mice.⁷² Selimi's group has previously identified BAI3 in a specific synapse of the cerebellum in adult mice.⁷³ BAI3 is highly expressed throughout brain development and is found in many neuronal populations including cerebellar Purkinje cells and hippocampal neurons, suggesting an involvement in brain development. Dendritogenesis and spinogenesis involve a remodeling of the actin cytoskeleton, in particular by Rho GTPases like Rac1. BAI1 is known to modulate the Rho GTPase Rac1 in nonneuronal cells through its interaction with the protein ELMO1.⁶⁹ In this context, they have shown that BAI3 is localized in actin-rich structures such as dendrites in cultured hippocampal neurons and that BAI3 knockdown in these neurons or in cultured Purkinje cells leads to an increase in the length of their dendritic arborization. Their data also revealed that the regulation of dendrite morphogenesis by BAI3 is partially dependent on BAI3 interacting with ELMO1 in cultured neurons.⁷⁴ To analyze the role of BAI3 *in vivo*, Sigoillot *et al.* first generated transgenic mice expressing a dominant-negative form of BAI3 specifically in Purkinje cells. Using this model, they showed that BAI3 controls dendritogenesis in a cell-autonomous manner. Using injection of recombinant lentiviral particles in the cerebellum of neonatal mice, they also showed that the knockdown of BAI3 in Purkinje cells leads to perturbations of dendrite differentiation and orientation.⁷⁴ The role of BAI3 in Purkinje cell spinogenesis and synaptogenesis was discussed because of its localization in parallel fiber/Purkinje cell synapses in the cerebellum. Given the link between BAI3 and some symptoms of psychiatric diseases, these data provide new insights into the study of neurodevelopmental disorders. Considering the diversity of expression exhibited by aGPCRs in the brain, BAI signaling pathways could serve as a prototype for a general mechanism regulating the maturation of neural networks.

Adhesion GPCRs in human developmental disorders

A variety of human developmental disorders can be directly linked to aGPCRs. The best known examples are BFPP and Usher syndrome, caused by mutations in *GPR56* and *VLGR1*, respectively.

Byoung-il Bae (Harvard Medical School). A postdoctoral fellow of Christopher A. Walsh (Harvard Medical School), Bae presented findings

on the regulation of *GPR56* expression in humans; previous work has shown that mutations in *GPR56* cause BFPP.^{48,49,75} As a number of potential functions are known for *GPR56* in diverse tissues and cell types, including muscle, melanoma, developing cerebral cortex, and immune cells,^{24,46,48,76} the question arises as to how *GPR56* expression is regulated in multiple contexts. Bae presented evidence indicating that human *GPR56* has at least 17 alternative promoters during evolution,⁷⁷ each of which drives spatially and temporally specific expression patterns. This contrasts with mouse *Gpr56*, which has only five promoters. Expansion of alternative promoters is partly achieved by retrotransposon insertions in the noncoding regions of the human genome. The work Bae described stems from the fortuitous discovery of a rare genetic disorder known as perisylvian polymicrogyria, which is caused by a 15-bp deletion mutation in a regulatory element of *GPR56*. The disorder disrupts human cortex surrounding the Sylvian fissure bilaterally (thus, *perisylvian* polymicrogyria) including Broca's area, the primary language area, by disrupting regional *GPR56* expression and blocking RFX transcription factor binding. *GPR56* expression levels regulate progenitor proliferation. *GPR56* splice

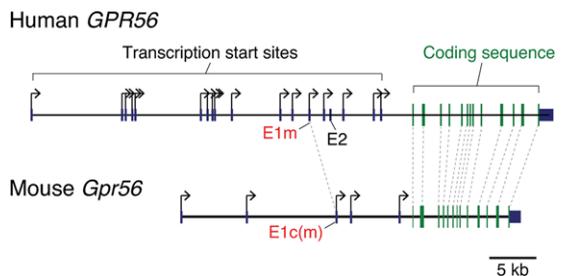


Figure 6. Multiple alternative transcription start sites allow fine control of temporal and spatial expression of *GPR56*. Human *GPR56* has at least 17 alternative transcription start sites, whereas mouse *Gpr56* has only five. Some of the transcription start sites arose as a result of retrotransposon insertions. For example, exon 1m, which causes perisylvian polymicrogyria when one of the upstream noncoding elements is mutated, is placental mammal-specific and shows homology at its 3' end to a long interspersed nuclear element (LINE), a family of retrotransposons; whereas noncoding exon 1m's noncoding element is conserved between humans and mice, another noncoding *GPR56* exon (exon 2) is present only in primates, derived from a primate-specific Alu retrotransposon. Comprehensive cataloging of RNA splice forms may soon suggest that complex expression patterns of other GPCRs may be regulated by alternative promoters. This figure is adapted from figure 4A in Ref. 78.

forms are highly variable between mice and humans (Fig. 6), which may explain how *GPR56* is expressed in diverse tissues and cell types, playing a unique role in each context. Alternative transcription start sites and promoters may be widely used in many genes,⁷⁸ including aGPCRs.

Uwe Wolfrum (Johannes Gutenberg University of Mainz). For several years, Wolfrum has been studying very large G protein-coupled receptor-1 (VLGR1) and associated clinical implications. VLGR1, also known as monogenetic audiogenic seizure susceptible 1 (MASS1) or GPR98, is by far the largest known GPCR.⁷⁹ *VLGR1* is expressed in numerous alternatively spliced isoforms, including VLGR1a and 1b. Mutations in human *VLGR1* cause the development of sensory-neural defects associated with Usher syndrome, the most common form of combined hereditary deaf-blindness.⁸⁰ Mouse *Vlgr1* mutants are characterized by the susceptibility to audiogenic seizures.⁸¹ A recent study by Shin *et al.*⁸² demonstrated that VLGR1 regulates myelin-associated protein (MAG) expression in myelinated regions of the murine brain via the heterotrimer G proteins $G\alpha_s$ and $G\alpha_q$ and the protein kinases PKA/PKC in response to changes in the extracellular Ca^{2+} concentration. Mutations in *Vlgr1* may cause the dysregulation of myelination and are responsible for epilepsy in *Vlgr1*-deficient mice. However, no evidence for a correlation between defects in *VLGR1* and epilepsy in humans has been reported. VLGR1 is a component of membrane adhesion complexes in inner ear hair cells and retinal photoreceptor cells. In both sensory systems, VLGR1 is found at the membrane adhesions of synapses. In addition, in hair cells, VLGR1 is part of the ankle-link complex essential for the correct development of mechanosensitive hair bundles. In photoreceptor cells, VLGR1 is a component of a periciliary protein network and is required for the assembly of fibrous links communicating between the membranes of the ciliary pocket.⁸³ In this compartment, VLGR1 may serve in modules associated with endocytosis and/or cargo delivery to the photoreceptor cilium.⁸⁴ These data have contributed significantly to our current insights into the functions of VLGR1 in the eye and the inner ear.

Adhesion GPCRs in cancer

With an ever-growing number of aGPCR members associated with cancer development and progres-

sion, understanding what roles they play and what influences them are essential. Three groups presented their research on aGPCRs in cancer.

Bigyan R. Bista (Massachusetts Institute of Technology). From Richard O. Hynes' lab (Massachusetts Institute of Technology), Bista discussed the involvement of GPR126 and CELSR2 in cancer development. They performed a systematic screen of all aGPCRs by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) in both murine and human normal non-transformed mammary cell lines and mammary carcinoma cell lines with varying metastatic properties, as well as tumor samples of varying grades from genetically engineered mouse tumor models. They identified several candidate genes for their possible roles in breast cancer progression and metastasis. On the basis of these analyses, and on cross-referencing them with the published gene expression data on human breast cancer cell lines and patient samples, *CELSR2* and *GPR126* were chosen for follow-up studies. To elucidate their functions in tumorigenesis and metastasis in breast cancer, Bista examined whether their expression is correlated with breast cancer progression and metastatic potential of breast cancer cell lines. He then explored the effects of their perturbations on tumor growth and metastasis by using RNA interference (RNAi) methods both *in vitro* and *in vivo*. Bista *et al.* are currently investigating the functions of these aGPCRs at the cellular and molecular levels (cell motility, migration, proliferation, and invasion, among other cancer-related properties) and also assessing the roles of the extracellular and intracellular domains of these receptors on downstream signaling pathways. Moreover, this group has also conducted orthotopic mammary transplants and tail-vein injections in mice to assay tumor growth, lung metastasis, and cell seeding and colonization in the lung. Their preliminary results suggest that, while GPR126 knockdown reduces the metastatic burden in an experimental metastasis model as well as an orthotopic transplant model of breast cancer, *CELSR2* knockdown does not affect the metastatic burden in an experimental metastasis model *in vivo*; however, *CELSR2* knockdown increases invadopodia formation in a matrix-degradation assay *in vitro*, hinting at roles for *CELSR2* in tumor invasion and intravasation at the primary tumor site. These findings uncover aGPCRs as novel candidates in cancer

progression, suggesting new roles for them in tumorigenesis and metastasis, and reveal diverse and contextual functions for this intriguing family of atypical aGPCRs.

Lei Xu (University of Rochester Medical Center).

Xu presented studies on the roles of GPR56 during melanoma progression. Her lab is interested in the impact of the ECM on cancer development. Accumulation and crosslinking of the ECM is a hallmark of cancer and actively promotes cancer progression.^{85,86} Removal of the ECM would thus have therapeutic benefits for cancer treatment, but this potential has not been actively pursued. Xu *et al.* previously showed that GPR56 was down-regulated in highly metastatic melanoma cells and its re-expression led to inhibition of metastasis and melanoma growth.²⁴ She also identified a ligand of GPR56, tissue transglutaminase (TG2), a crosslinking enzyme in the ECM that functions to stabilize the ECM and in cell adhesion.⁸⁷ The binding between GPR56 and TG2 suggested that GPR56 might inhibit melanoma growth and metastasis via TG2-mediated ECM remodeling. Xenograft studies using immunodeficient *Tg2^{-/-}* mice showed that

GPR56 antagonizes the tumor-promoting function of TG2 in melanoma.⁸⁸ To understand the mechanism of this antagonism, biochemical analyses were performed and revealed that GPR56 removes TG2 from the surface of melanoma cells via receptor-mediated endocytosis and degradation. This loss of TG2 by GPR56 led to a reduction in fibronectin deposition and accumulation of focal adhesion kinase. Consistent with this, expression of GPR56 inhibited melanoma cell adhesion on fibronectin. This suppression of cell adhesion by GPR56 may explain Xu's earlier findings, which delineate an axis of negative regulation from GPR56, to PKC α activation, to VEGF secretion, and ultimately to angiogenesis and melanoma growth.⁸⁹ Cell-ECM adhesion has been shown to activate PKC α . Thus, it is possible that the impaired ECM deposition upon GPR56 expression results in a reduction in cell adhesion, which in turn impedes the activation of PKC α in melanoma cells (Fig. 7). Together, the above findings suggest that GPR56 inhibits melanoma progression by removing TG2 in the ECM, and, consequently, ECM removal via receptor-mediated endocytosis may be utilized as a therapeutic strategy for cancer treatment. GPR56, and perhaps other aGPCRs, could

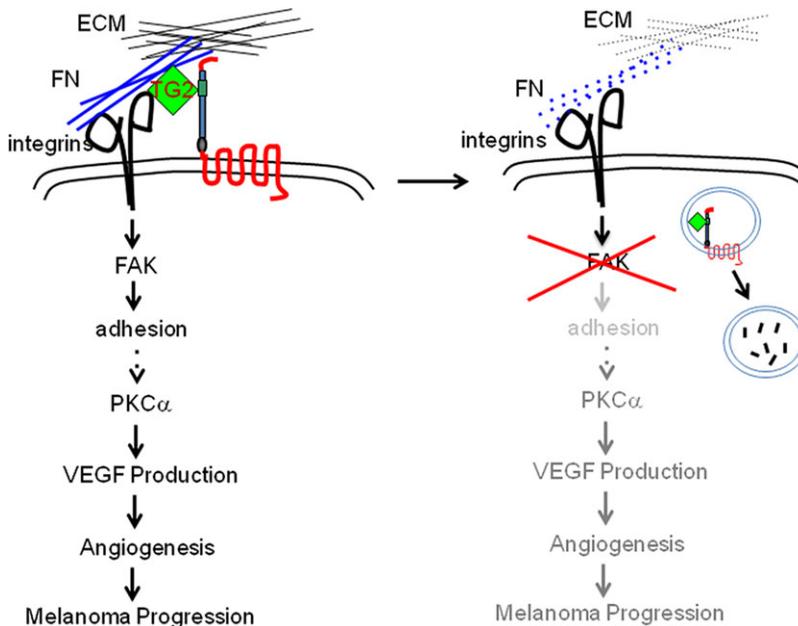


Figure 7. GPR56 inhibits melanoma progression by removing TG2 in the ECM. In melanoma cells, GPR56 internalizes TG2 via endocytosis, leading to its degradation in lysosomes. The loss of TG2 is associated with destabilized fibronectin deposition in the ECM and compromised cell adhesion, which might result in inactivation of PKC α and subsequent impairment of VEGF secretion and angiogenesis, and ultimately inhibition of melanoma progression.

potentially be targets for such ECM-based cancer therapy.

Mingyao Liu (East China Normal University).

Liu reported on GPR116 as a new regulator in cancer progression. Liu and colleagues hypothesized that aGPCRs are potentially involved in cancer metastasis. After expression and functional screening of the aGPCR family in breast cancer cells, Liu and colleagues identified GPR116 as a novel regulator of breast cancer metastasis.⁹⁰ In their study, they found that knockdown of *Gpr116* in highly metastatic (MDA-MB-231) breast cancer cells suppressed cell migration and invasion. Conversely, ectopic *Gpr116* expression in poorly metastatic (MCF-7 and Hs578T) cells promoted cell invasion. Liu further showed that knockdown of *Gpr116* inhibited breast cancer cell metastasis in two mammary tumor metastasis mouse models (Fig. 8). Moreover, GPR116 modulated the formation of lamellipodia and actin stress fibers in cells in a RhoA- and Rac1-

dependent manner. At the molecular level, GPR116 regulated cell motility and morphology through the G_{α_q} -p63RhoGEF-RhoA/Rac1 pathway. The biological significance of GPR116 in breast cancer is substantiated in human patient samples, where *GPR116* expression is significantly correlated with breast tumor progression, recurrence, and poor prognosis. These findings show that GPR116 is crucial for the metastasis of breast cancer and support GPR116 as a potential prognostic marker and drug target in metastatic human breast cancer.

New roles for “old” aGPCRs

Members of the EGF-like subfamily were among the first aGPCRs to be functionally characterized, with a special focus on their role in immune defense and leukocyte adhesion. Two groups have introduced physiological relevance for these previously identified receptors in previously unrelated research fields. Moreover, the new role of GPR56 in natural killer (NK) cell function was discussed by two independent groups.

Gabriele Aust (University of Leipzig).

Aust *et al.* published the first study elucidating involvement of an aGPCR in the regulation of epithelial ion transport. CD97 is expressed at the luminal membrane and in adherens junctions of enterocytes and shows an expression gradient along the crypt-villus axis in the normal human intestine.^{91–93} To examine CD97-dependent intestinal function, they verified whether CD97 influences intestinal barrier properties. Transgenic mice overexpressing CD97 in intestinal epithelial cells⁹² showed normal total transepithelial, epithelial, and subepithelial resistance, as well as paracellular permeabilities comparable to wild-type mice. Stimulated intestinal chloride secretion was increased in *Cd97*-transgenic compared to wild-type mice. The increase depends on the 7TM and/or intracellular domain of CD97, because mice overexpressing C-terminal truncated CD97 in enterocytes⁹³ did not show enhanced stimulated chloride secretion. To verify the specificity of this enhancement, they generated stable *CD97* and *CD97*-scrambled siRNA clones of human colorectal cells, which established a tight epithelium with high total transepithelial resistance. The decrease of CD97 nearly abrogated stimulated chloride secretion. Messenger RNA microarrays of several clones confirmed the downregulation of an intestinal chloride channel by CD97, which plays an important

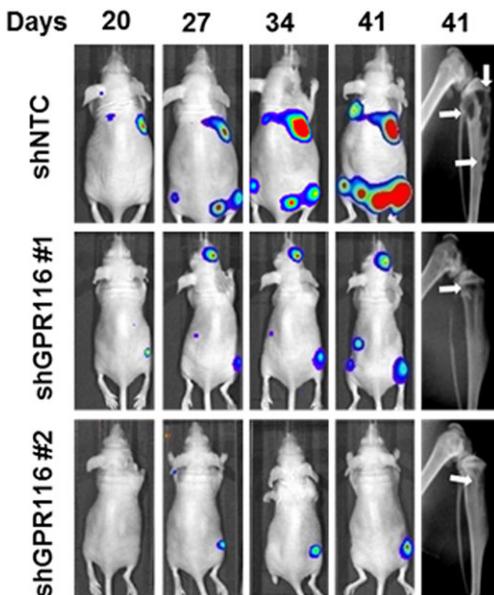


Figure 8. *Gpr116* knockdown inhibits breast cancer cell bone metastasis in a mouse tumor metastasis model. Two shRNA-mediated stable *Gpr116* knockdown MDA-MB-231 cells (shGPR116 #1 and #2) or control cells (shNTC) (2×10^5 cells) were injected directly into the left ventricle of 4- to 5-week-old female *nu/nu* mice ($n = 10$ mice in each group). Bioluminescent (left) and radiographic (right) imaging of representative mice in each experimental group at the indicated days are shown in the same color scale. White arrows indicate osteolytic lesions (right).

role in clinical physiology and pathology. These data show that, in enterocytes, CD97 alters chloride transport and establishes an osmotic gradient enabling intestinal fluid secretion. This is pivotal in the creation of an ideal environment for effective enzymatic digestion, nutrient absorption, and stool movement.

Helen Song (Massachusetts Eye and Ear Infirmary). A graduate student of Joan Stein-Streilein (Massachusetts Eye and Ear Infirmary), Song presented studies on the involvement of EMR2 in immune responses in the eye. In the mouse, the macrophage aGPCR molecule, F4/80, is required for the development of regulatory T (T_{reg}) cells in two models of immune privilege, the eye and gut.⁹⁴ Since F4/80 is not expressed in humans, the purpose of this research was to determine the human analog of F4/80. Belonging to the EGF-TM7 family, F4/80 shares much of its sequence homology with other family members, including EMR molecules in humans. EMR1 is the structural ortholog of F4/80⁹⁵ and EMR2 has shown immune-suppressing function in tumor cells.⁹⁶ Thus, Song and colleagues investigated the possible suppressor role of the EMR family in human ocular tolerance.

To characterize the antigen-presenting cells (APCs), Song and colleagues treated fresh human peripheral blood monocytes with immunosuppressive aqueous humor factor TGF- β and with a foreign antigen, tetanus toxoid (TT), and measured APC surface markers. Analyzed by flow cytometry, CD14⁺ APCs expressed upregulated PD-L1, ILT3, and EMR2, PD-L1 and ILT3 being tolerogenic receptors that downregulate immune responses. APCs did not express increased levels of CD40 and CD97. Additionally, tolerogenic APCs (tolAPC) were cocultured with autologous CD3⁺ lymphocytes to simulate the cellular mechanisms of ocular tolerance, termed ACAID (anterior chamber-associated immune deviations) in human cells.⁹⁷ This co-culture induced a population of CD4⁺ CD25⁺ FOXP3⁺ T_{reg} cells that showed an immunosuppressive cytokine profile with increased IL-10 production and decreased interferon (IFN)- γ production.

To further analyze the function of TGF- β -generated T_{reg} cells, a humanized NOD SCID mouse model was used. Immunocompromised mice were injected with human peripheral blood mononucleated cells and immunized with TT and complete Fre-

und's adjuvant. A week later, mice were challenged locally with TT in the ear pinnae, and ear swelling was measured as a function of the delayed-type hypersensitivity (DTH) response. T_{reg} cells that were incubated with EMR2⁺ APCs were able to suppress the DTH swelling response. Blocking EMR2 expression with soluble chondroitin sulfate abrogates the induction of the T_{reg} population. These data suggest that EMR2⁺ tolerogenic APCs generate T_{reg} cells specific to a foreign antigen. Human T_{reg} cells injected into an SCID mouse are able to suppress inflammation *in vivo*, attesting to their tolerogenic function. In the absence of EMR2 on the APCs, these T_{reg} cells are not induced, indicating a possible requirement of EMR2 in the generation of immune tolerance in humans.

Jörg Hamann (University of Amsterdam) and Hsi-Hsien Lin (Chang Gung University). Hamann and Lin previously showed that an aGPCR gene cluster comprising GPR56, GPR97, and GPR114 is expressed in human immune cells.^{76,98} NK cells are important innate immune lymphoid cells and act as a first line of defense against virus-infected cells and tumor cells. GPR56 is strongly expressed by mature CD56^{dim} NK cells, but little if any expression is detected in less mature CD56^{bright} NK cells.⁷⁶ CD56^{dim} NK cells stably express GPR56 independent of their further differentiation toward stages of senescence and, finally, exhaustion indicated by the loss of CD94 expression and the stepwise acquisition of KLRG1, CD57, and PD-1 expression. As IL-2, an essential growth factor for NK cells, negatively regulates the expression of GPR56 in primary NK cells, the NK-92 cell line—cells that are IL-2 dependent and express very little GPR56, but upregulate GPR56 expression significantly when IL-2 is withdrawn—was used to investigate the transcriptional regulation and cellular function of GPR56. IL-2 withdrawal resulted in changes in the expression of transcription factors, including BCL-6, Blimp, T-BET, and Eomes. Using short hairpin RNA (shRNA) knockdown in NK-92 cells, a candidate factor potentially involved in the expression of GPR56 was identified. Notably, this transcription factor was not expressed in CD56^{bright} primary NK cells. In other experiments, NK-92 cells stably overexpressing GPR56 were established using a retroviral expression system. NK-92-GPR56 cells expressed less granzyme B at steady state and lower levels of TNF- α and IFN- γ when activated



Figure 9. Participants of the 7th International Adhesion GPCR Workshop in the Folkman Auditorium, Boston Children's Hospital, Harvard Medical School, Boston.

by phorbol 12-myristate 13-acetate (PMA), suggesting diminished NK effector functions. Examination of various mechanisms involved in cellular cytotoxicity revealed reduced target cell conjugation, degranulation, and killing in NK-92 cells that overexpress GPR56. GPR56 was previously shown to associate with the tetraspanin CD81,⁹⁹ which has been demonstrated to inhibit NK cell function when cross-linked by immobilized antibodies or ligand. NK-92–GPR56 cells expressed lower levels of CD81 when compared with NK-92–Neo cells. The expression levels of GPR56 and CD81 were reduced even further when cultured with target cells, suggesting dynamic regulation of the receptors. The functional significance of the GPR56–CD81 receptor complex on NK cell biology requires further investigation.

Previous studies showed that PMA causes downregulation of GPR56 in U937 cells.⁹⁹ Unpublished work from Lin's and Hamann's groups showed that, in NK-92 cells, PMA addition results in rapid downregulation of GPR56 through protein kinase C (PKC)-dependent receptor internalization. These observations raised the possibilities that GPR56 is downregulated owing to either ligand-mediated desensitization or as a consequence of NK cell activation. Importantly, contact with the known GPR56 binding partners, collagen III and tissue transglutaminase 2, did not result in downregulation of the receptor. Under physiological conditions, cytokines, target cells, and NK receptors can activate NK cells. Evidence was obtained that NK cell activation caused PKC-dependent downregulation of GPR56 primarily through shedding of its extracellular domain (unpublished).

Conclusions

The 7th International Adhesion GPCR Workshop (a photograph of workshop participants is shown in Fig. 9) concluded with several new advancements and hypotheses. The field has made tremendous progress toward an understanding of how aGPCRs signal *in vitro* and *in vivo*. The discovery of new ligands was presented, as well as new activating mechanisms. It seems that the early-postulated dual role of these proteins is even more complex than anticipated; aGPCRs are capable of not only adhesion and autologous signal transduction, but rather of adhesion, mechanosensation, and autologous and heterologous signal transmission. In a physiological context, aGPCRs participate in a multitude of developmental processes and pathological conditions. Besides their well-established roles in immune defense, neural development, and cancer, new roles in muscle function, chloride secretion, and other physiological processes have recently been reported. Given that there are still many members of the aGPCR family about which little is known beyond their basic expression patterns, the aGPCR field will likely continue to expand for some time to come. The next International Adhesion GPCR Workshop, to be held in Leipzig, Germany in 2016, will undoubtedly provide more in-depth characterization of the presented findings.

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Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix S1.

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