Type IV collagen is an activating ligand for the adhesion G protein–coupled receptor GPR126

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GPR126 is an orphan heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptor (GPCR) that is essential for the development of diverse organs. We found that type IV collagen, a major constituent of the basement membrane, binds to GPR126 and activates its signaling function. Type IV collagen stimulated the production of cyclic adenosine monophosphate in rodent Schwann cells, which require GPR126 activity to differentiate, and in human embryonic kidney (HEK) 293 cells expressing exogenous GPR126. Type IV collagen specifically bound to the extracellular amino-terminal region of GPR126 containing the CUB (complement, Uegf, Bmp1) and pentraxin domains. GPR126 derivatives lacking the entire amino-terminal region were constitutively active, suggesting that this region inhibits signaling and that ligand binding relieves this inhibition to stimulate receptor activity. A new zebrafish mutation that truncates GPR126 after the CUB and pentraxin domains disrupted development of peripheral nerves and the inner ear. Thus, our findings identify type IV collagen as an activating ligand for GPR126, define its mechanism of activation, and highlight a previously unrecognized signaling function of type IV collagen in basement membranes.

INTRODUCTION

Receptors on the cell surface control a myriad of processes in development, physiology, and behavior. G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors (GPCRs) have a characteristic heptahelical transmembrane [seven-transmembrane (7TM)] region, and they comprise the largest group of receptors in vertebrates (1). GPCRs receive diverse inputs, such as photons, small molecules, neuropeptides, and proteins (2). Agonists induce a conformational change in GPCRs that activates the associated heterotrimeric G proteins (3). Different GPCRs are coupled to distinct G proteins that activate specific downstream second-messenger systems by binding to effector proteins, such as adenyl cyclases and phospholipase C (4). Reflecting the diversity of their ligands and downstream effectors, GPCRs have an enormous number of critical functions in receiving signals between neighboring and distant cells, and in sensing environmental cues through olfaction, taste, and vision. In addition, about 30% of all U.S. Food and Drug Administration–approved drugs specifically target GPCRs (5). Many GPCRs remain “orphan” receptors with no known activating ligands, and there is intense interest in defining the functions of these receptors and their signaling pathways.

The adhesion GPCRs (aGPCRs) are a recently defined subfamily that combines characteristics of adhesion molecules and 7TM receptors (6). The aGPCRs share with other GPCRs the heptahelical domain, but are distinguished by long, heavily glycosylated N-terminal regions that contain different adhesion domains, such as cadherin, epidermal growth factor (EGF), and thrombospondin motifs. A hallmark of the aGPCR family is the presence of a conserved GPCR proteolytic site (GPS) within the GPCR autoproteolysis–inducing (GAIN) domain (7, 8). The GAIN domain is always located in the extracellular region, just N-terminal to the first of the 7TM helices. During receptor processing, autoproteolysis in the GAIN domain cleaves aGPCRs into N- and C-terminal regions that remain noncovalently associated at the cell surface (7, 9–11). Three distinct models have been proposed to explain the role of the GAIN domain in aGPCR activation. In the tethered inverse agonist model, the cleaved N-terminal region interacts with the 7TM region to antagonize receptor signaling; the receptor is activated when a ligand binds the N-terminal region to relieve this antagonism. The main evidence for this model derives from studies showing that deletion constructs lacking the N-terminal region are constitutively active receptors, as has been reported for the aGPCRs GPR56 (12, 13), CD97 (14), BAII2 (15), and BAII1 (16). Analysis of the aGPCR latrophilin-1 in the nematode led to the tethered agonist model, in which ligand binding allows the GAIN domain to interact with the 7TM region in a way that activates receptor signaling (17). A third model, based on work on the aGPCR CD97, proposes that the N-terminal region binds to activating ligands and that GAIN domain autoproteolysis leads to removal of the N-terminal region and elimination of signaling (18). Finally, there is also emerging evidence that the N-terminal regions of some aGPCRs, cleaved at the GAIN domain or in other regions, may act as soluble factors with other activities, independent of G protein activation (16, 17).

Most aGPCRs are orphan receptors. Many studies have identified proteins that bind to different aGPCRs, but most of these are not known to activate receptor signaling, and many may have a purely adhesive function (10). For example, the aGPCR latrophilin-1 has three known classes of high-affinity ligands [teneurin-2 (19), FLRT (fibronectin leucine-rich transmembrane) proteins (20), and the neurexin proteins (21)], but receptor activation has not been observed upon ligand treatment. The best-defined aGPCR-ligand pair is type III collagen and GPR56. Type III collagen can bind to GPR56 and activate Go12/13 to control neuronal progenitor migration (22). Moreover, type III collagen knockout mice exhibit an abnormal cortical development phenotype similar to that of GPR56 mutants (23), and mutations in the N-terminal region of GPR56 that cause disease in humans can prevent binding to type III collagen (24). Identification of activating ligands for other aGPCRs will elucidate their functions and enable systematic studies to resolve the various models of aGPCR activation.

Analyses in zebrafish and mice have defined Gpr126 as an orphan aGPCR that functions in diverse essential processes, including the
myelination of peripheral nerves, trabeculation of the developing heart, and formation of the semicircular canals of the inner ear. A zebrafish screen for disruptions in myelin formation discovered mutations in gpr126 (25, 26). Analysis of the zebrafish mutant phenotype revealed that gpr126 is essential for Schwann cells to initiate myelin formation, and subsequent characterization of knockout mice demonstrated that this function is conserved in mammals (26, 27). Treatment of mutant zebrafish for forskolin to increase cAMP (adenosine 3',5'-monophosphate) rescues the mutant phenotype (26, 28), providing evidence that Gpr126 may signal through a cAMP second-messenger cascade. Additional studies provide evidence that Gpr126 signals through a pathway involving G\(\alpha_s\), adenylyl cyclases, and protein kinase A (PKA) (28, 29). Zebrafish gpr126 mutants also have defects in morphogenesis of the semicircular canals in the inner ear, and this phenotype could also be rescued by increasing cAMP (30). In addition to defects in Schwann cell development and myelination, Gpr126 mutant mice have abnormalities in the developing heart, and they die during embryogenesis or shortly after birth (27, 31, 32). A recent study using morpholinos in zebrafish reported that targeting the translation start site of Gpr126 disrupted heart development, ear development, and myelination, whereas a morpholino that blocked splicing of a downstream exon caused only myelination and ear defects that are characteristic of previously characterized gpr126 mutants (32). The heart defects were rescued by a construct encoding most of the N-terminal region of Gpr126, but not the 7TM region (32). This N-terminal construct did not rescue the inner ear or myelination phenotypes. The authors suggested that heart development requires a function of the Gpr126 N-terminal region that is independent of G protein signaling, but the domains involved and the mechanism of action were not defined.

Here, we identified type IV collagen as an activating ligand for Gpr126 and defined the activation mechanism for this essential gGPCR, highlighting a previously unrecognized signaling function of type IV collagen in basement membranes.

RESULTS

Gpr126 binds specifically to type IV collagen

To identify ligands for Gpr126, we expressed a secreted Fc-tagged protein containing the N-terminal fragment of zebrafish Gpr126 (Gpr126-NT-Fc, Fig. 1A) in human embryonic kidney (HEK) 293 cells. Previous studies show that Gpr126 and type IV collagen in the extracellular matrix (ECM) are involved in myelination (26, 33–35), and a recent paper identified type III collagen as an activating ligand for Gpr56 (22). To search for specific interactions between Gpr126 and different collagen proteins, we performed a series of pull-down assays with the Gpr126 N-terminal Fc fusion protein and purified collagen proteins, types I, II, III, IV, and V. Biotinylated collagen proteins were incubated with streptavidin-agarose and conditioned medium from transfected HEK293 cells expressing Gpr126-NT-Fc. Bound proteins were analyzed by immunoblot with an antibody specific to Fc to determine whether any collagen proteins interacted with the Gpr126 N-terminal region. Gpr126-NT-Fc robustly interacted with type IV collagen, but not the other four collagen types (Fig. 1B). A control protein representing only the Fc tag of the fusion protein did not bind to any of the collagen proteins or to bovine serum albumin (BSA) (fig. S1). In reciprocal experiments, biotinylated collagen proteins and medium containing Gpr126-NT-Fc were incubated with protein A–agarose. Again, Gpr126-NT-Fc interacted robustly with type IV collagen, but not with the other four collagen types or BSA (Fig. 1D). These results demonstrate that the N-terminal region of Gpr126 specifically binds to type IV collagen.
The N-terminal region of Gpr126 contains several conserved elements, including the CUB, PTX, hormone-binding, and GAIN domains (39, 40). We focused on the CUB and PTX domains because these domains in other proteins can bind to various collagen-like proteins, including type IV collagen (41–44). We analyzed the binding activity of two complementary C fusion proteins (Fig. 3A). CUBPTX-Fc contained a signal peptide, the CUB domain, and the PTX domain followed by an Fc tag (amino acids 41 to 336 of Gpr126). The complementary protein, ΔCUBPTX-Fc, contained a signal peptide, the rest of the Gpr126 N-terminal region, and an Fc tag (amino acids 337 to 827 of Gpr126). The fusion proteins were harvested from conditioned medium of transfected HEK293 cells and analyzed in pull-down experiments with biotinylated collagen type IV and streptavidin-agarose. Both Gpr126-NT-Fc and CUBPTX-Fc strongly bound collagen type IV, in contrast to ΔCUBPTX-Fc, which displayed no detectable interaction (Fig. 3B).

To quantitatively compare the affinities of Gpr126-NT-Fc and CUBPTX-Fc for type IV collagen, we used surface plasmon resonance (SPR) binding assays. Collagen type IV was coupled to the surface of the sensor chip, and the affinity-purified Gpr126-NT-Fc protein was introduced at various concentrations, with the surface being regenerated between trials. Gpr126-NT-Fc bound to collagen IV with high affinity in duplicate trials (Fig. 3C). The mean calculated affinity constant (KD) value was 1.3 nM, similar to the EC50 for activation (Fig. 2D), with association and dissociation constants of 3.06 × 10^(-1) ± 0.05 × 10^(-2) (1/ms) and 4.04 × 10^(-4) ± 0.8 × 10^(-4) (1/s), respectively. In similar experiments, CUBPTX-Fc also bound to collagen IV with high affinity in duplicate trials (Fig. 3D). The mean calculated KD value was 1.2 nM, with association and dissociation constants of 2.78 × 10^(-3) ± 0.4 × 10^(-3) (1/s) and 3.33 × 10^(-3) ± 1 × 10^(-3) (1/s), respectively. These results indicate that the region of Gpr126 containing the CUB and PTX domains binds type IV collagen with high affinity and specificity.

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The N-terminal region of Gpr126 controls its signaling activity

Functional studies of aGPCRs have led to different models of the role of the N-terminal region in receptor activation (12–17, 45). One model is that the cleaved N-terminal region antagonizes receptor signaling activity, so that truncation of the N terminus can create a constitutively active receptor (12–16). To test whether this scenario might apply to Gpr126, we created FLAGagged Gpr126 constructs representing the full-length and N-terminally cleaved receptor (Fig. 4A). Both constructs were expressed at similar amounts, as indicated by immunostaining of transfected cells with an antibody against FLAG (Fig. S2). In lysates of transfected cells (Fig. 4B, right lanes), analysis of Gpr126-FLAG detected multiple bands, including a 35-kd protein corresponding to the C-terminal region after GPS cleavage (bottom arrow), and higher–molecular weight species representing modified and uncleaved forms of Gpr126. Multiple proteins were also detected in samples from cells transfected with Gpr126NT-FLAG (Fig. 4B), including the 35-kd protein and an abundant species with an apparent molecular weight of about 60 kD (top arrow). The 60-kd protein could be a ubiquitinated form, which is observed in the active species of other GPCRs, including the aGPCR GPR56 (12, 46, 47). We also determined the extent of surface expression for each construct using a standard surface

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A region of Gpr126 containing CUB and PTX domains mediates the high-affinity interaction with type IV collagen. (A) Schematic diagram depicting truncated versions of the Gpr126-NT-Fc fusion protein either containing (CUBPTX-Fc) or lacking (ΔCUBΔPTX-Fc) the CUB and PTX domains. (B) Pull-down experiments using conditioned medium containing Gpr126-NT-Fc, ΔCUBΔPTX-Fc, or CUBPTX-Fc incubated with biotinylated type IV collagen and streptavidin-agarose. Blot is representative of four experiments. (C and D) Biacore sensograms detecting the interaction of type IV collagen with Gpr126-NT-Fc and CUBPTX-Fc. SPR monitored the binding of various concentrations of fusion proteins to immobilized collagen IV on a biosensor chip. The association and dissociation constants (K_a and K_d, respectively) for each trial are shown in the figure panels. Data are representative of two experiments; the values for association and dissociation constants are reported as means ± SD in the text.

The gpr126<sup>186</sup> mutation truncates Gpr126 after the CUB and PTX domains and disrupts myelination and inner ear development, but not the heart

In a genetic screen for mutations that disrupt myelinated axons, we recovered a new allele of gpr126, gpr126<sup>186</sup>, that introduces a premature stop codon at position 406 and truncates the protein between the PTX and hormone-binding domains (Fig. 5, A and B). The amount of gpr126 mRNA was similar in wild-type and gpr126<sup>186/186</sup> homozygotes, and therefore, it is possible that the mutant allele produced a truncated protein containing the CUB and PTX domains (fig. S3). Like other strong mutant
alleles of gpr126, homozygous gpr126st86/st86 mutants lack expression of mbp, encoding myelin basic protein, in Schwann cells and have swollen ears (Fig. 5, C and D). Heart development appeared to be normal in gpr126st86/st86 mutants (Fig. 5D). Like gpr126st49/st49 mutants (26, 28), homozygous gpr126st86/st86 mutants survived to adulthood, further confirming that the mutation did not cause lethal heart abnormalities and allowing us to examine the possible maternal functions of gpr126. To examine the phenotype caused by loss of maternal (M) and zygotic (Z) function, we crossed a heterozygous gpr126st86/+ male to a gpr126st86/st86 homozygous mutant female. The resulting Mgpr126st86/+ mutants had a wild-type phenotype, and MZgpr126st86/st86 mutants resembled Zgpr126st86/st86 mutants, having swollen ears and normal hearts (Fig. 5D). If the previous proposal is correct that a region of Gpr126 has a function in heart development in zebrafish (32), our analysis suggests that this function is fulfilled by a protein fragment containing the first 405 amino acids of Gpr126—the region encoded 5′ to the premature stop codon in the gpr126st86 mutation.

**DISCUSSION**

Our analysis indicates that type IV collagen is a specific agonist of Gpr126. Type IV collagen bound to the N-terminal region of Gpr126 with high affinity and high specificity, unlike the other collagen proteins tested. The region of Gpr126 containing the adjacent CUB and PTX domains mediated the interaction with type IV collagen. Treatment of Schwann cells and Gpr126-expressing HEK293 cells with type IV collagen stimulated the production of cAMP, providing evidence that type IV collagen activates Gpr126 signaling. Finally, constructs lacking the N-terminal region of Gpr126 were constitutively active, providing evidence that the N-terminal region antagonizes receptor signaling activity and that type IV collagen binding relieves this repression to trigger downstream signaling.

In combination with previous work, our studies provide strong evidence that type IV collagen is an endogenous activating ligand for Gpr126. Type IV collagen is a component of the ECM in nerves, heart, and the inner ear at stages when Gpr126 is required for the development of these organs (48–50). In particular, previous work supports a role for type IV collagen in the myelination of peripheral nerves, which is the best-defined function of Gpr126 in fish and mammals (26, 27). After they associate with axons, but before myelination, Schwann cells secrete type IV collagen, laminin, and other components to form a continuous basal lamina (35, 51). Classic studies using Schwann cell and neuron cocultures demonstrate that Schwann cell myelination is dependent on the secretion of type IV collagen and proper formation of the basal lamina (52, 53). For example, Schwann cells cultured with neurons in medium lacking ascorbate proliferate, but do not form myelin (53). Addition of ascorbate, a cofactor necessary for the post-translational modification and secretion of collagen, promotes type IV collagen secretion, basal lamina formation, and myelination (53). Addition of a reconstituted matrix, composed mainly of collagen type IV and laminin, to cultures lacking ascorbate also induces Schwann cells to ensheath axons, produce myelin proteins, and generate myelin (54). Addition of type I collagen does not promote myelination in these cultures, demonstrating the specificity of the requirement for type IV collagen—an observation that parallels our analysis of the specificity of the interaction between type IV collagen and Gpr126.

Our analysis, together with previous studies showing that Schwann cells synthesize the basal lamina that is essential for their myelination, suggests that type IV collagen serves as an autocrine signal that promotes myelination. Just before myelination, promyelinating Schwann cells associate with the axon on one surface, and the basal lamina on the other. This polarization is essential for myelination (55). Our analysis suggests that type IV collagen signaling occurs to initiate myelination after the ECM is established. In addition to the ECM, axonal signals, including Ngf1–type III, are needed to promote myelination (36). In light of previous work showing that changes in cAMP affect Schwann cell’s response to Ngf1 (57), an attractive possibility is that activation of Gpr126 by type IV collagen changes the response of the Schwann cell so that axonal neuregulin signals trigger myelination instead of proliferation, as occurs at earlier stages.

The GAIN domain is the main structural feature that unites the aGPCRs and distinguishes them from other GPCRs. Nonetheless, the role
of the GAIN domain and the autoproteolytic cleavage that it mediates is so far unclear. Our results demonstrate that removal of the N-terminal region of Gpr126 leads to robust, ligand-independent signaling. Thus, our work on Gpr126 parallels studies of GPR56 (12, 13), BAI1 (15), BAI1 (16), and CD97 (14), which are also constitutively activated upon deletion of the region from the GAIN cleavage to the N terminus. Collectively, these studies support the tethered inverse agonist model for aGPCR signaling, in which the N-terminal region suppresses signaling in a way that is relieved upon ligand binding. Binding of the activating ligand may involve removal of the cleaved N-terminal region or alteration of its interaction with the 7TM domain. A mode of activation that involves cleaving the peptide backbone may lead to irreversible activation of the receptor that persists even after the ligand is no longer present, similar to the activation of the GPCR protease-activated receptor 1 (PAR-1) by the protease thrombin (58). Our data, however, showed that Gpr126 signaling is transient, with cAMP concentration peaking 1 to 2 hours after addition of type IV collagen and then declining. As in the case of GPR56 (12), it is possible that Gpr126 signaling is inactivated by interaction with β-arrestin 2 and ubiquitination. Prolonged stimulation ofGPCRs with ligand can also cause receptor internalization, many times facilitated by the arrestin proteins, leading to signal termination (18, 59, 60). It is also possible that, like the β2-adrenergic receptor (β2AR), Gpr126 signaling initiates a feedback loop in which PKA phosphorylates the receptor, weakening its association with Gi and strengthening its association with Gs, which inhibits adenylyl cyclase and reduces cAMP (61). Like the β2AR, Gpr126 has predicted PKA phosphorylation sites in the third intracellular loop and the C-terminal tail, and there is also evidence that Gpr126 couples to Gi and Gs (29). We propose that type IV collagen binding to Gpr126 initially activates only Gi and that Gi only comes into play to attenuate signaling after increased or extended action of Gi. This would explain the transient rise of cAMP that begins shortly after addition of type IV collagen (Fig. 2C) and the reduction of cAMP in Gpr126 mutant nerves (29). The transient nature of Gpr126 signaling is also supported by our previous study showing that Gpr126 is only required at the onset of myelination, so that myelin maturation and maintenance can proceed independently of Gpr126 signaling (28).

There is emerging evidence that the cleaved N-terminal regions of some aGPCRs may have functions independent of the 7TM. For example, the cleaved N-terminal fragment of BAI1 (vasculostatin) can inhibit angiogenesis by binding integrins to disrupt migration and proliferation of endothelial cells (62, 63). Similarly, a recent study proposed that an N-terminal fragment of Gpr126 has a function that is independent of the 7TM domain and is required for heart development in zebrafish (32). Although this function is proposed to be distinct from the Gαi/cAMP pathway that acts in myelination and inner ear development, the mechanism by which an N-terminal fragment might act in heart development has not been defined. The precise domains of Gpr126 involved in the putative heart function have also not been defined, but analysis of truncation constructs showed that a protein containing the first 783 amino acids of Gpr126 could rescue heart defects in some gpr126 morphant embryos, but not myelination defects (32). In the same study, a construct containing amino acids 1 to 572 did not rescue the heart defects, implicating the GAIN and hormone-binding domains in this putative heart function (32). In contrast, our analysis of a new mutation in gpr126 shows that animals with a premature stop codon upstream of the hormone-binding and GAIN domains resemble previously characterized gpr126 mutant alleles, with myelination and ear defects, but normal heart development. Thus, our analysis suggests that if the N-terminal region of Gpr126 has a function in heart development, it is mediated by the region containing the CUB and PTX domains. We have shown that this region of Gpr126 binds to type IV collagen with high specificity and affinity, suggesting that any 7TM-independent function of this N-terminal fragment may involve collagen binding. To further address the previous proposal that this region has a 7TM-independent function in heart development, it will be important to confirm the morpholoins studies with corresponding mutations in gpr126.

Together with previous studies, our analysis of the interaction between type IV collagen and Gpr126 emphasizes the close relationship between aGPCRs and components of the ECM. On the basis of sequence similarity of the 7TM domain, Gpr126 and human GPR56 belong to the same subclass of aGPCR (6), collagen proteins activate both receptors (22), and deletion of the N-terminal region leads to ligand-independent signaling in both cases (12, 13). There are, however, important differences between Gpr126 and GPR56 and their collagen ligands. Type IV collagen forms a meshwork, whereas type III forms fibrils (64), Gpr126 and GPR56 couple to different G proteins and activate different intracellular second messengers (65), and type IV collagen acts as an autocrine signal for Gpr126-expressing Schwann cells, whereas type III collagen is a paracrine signal for migrating neurons expressing GPR56 (22). By defining type IV collagen as an activating ligand for Gpr126, our results have increased the understanding of aGPCRs and highlighted an important signaling function of the ECM that may apply to other members of this versatile family of GPCRs and their functions in diverse processes throughout the organism.

MATERIALS AND METHODS

Constructs

The Gpr126-NT-Fc was cloned into pFUSE-hlgG2-Fc2 vector (InvivoGen) using homologous recombination method with the GenScript CloneEZ Kit per the manufacturer's protocol. Target DNA was amplified by polymerase chain reaction (PCR) [primers: 5′-GTCAGAATTCGTCCATCGGCACTGGTCTGTTTACGAATTCGTCCATCGGCACTGGTCTGTTTACGAATTCGTCCATCGGGTTCAAGGTAAGATACT-3′ (forward) and 5′-GTCAGAATTCGTCCATCGGCACTGGTCTGTTTACGAATTCGTCCATCGGCACTGGTCTGTTTACGAATTCGTCCATCGGGTTCAAGGTAAGATACT-3′ (reverse)] and inserted into pBGP2X-Fc and pACBPTX-Fc [primers: 5′-CCATGGTTCAGAGCTGCCAGAGTAGCACC-3′ (forward) and 5′-CCATGGTTCAGAGCTGCCAGAGTAGCACC-3′ (reverse)]. C-terminal FLAG tag Gpr126 was cloned into p3XFLAG-CMV-14 vector (Sigma-Aldrich) using standard cloning techniques and restriction sites Hind III and Cla I [primers: 5′-AAATTTAAGCTTATGATGATTGCTTTCGCACTGCAT-3′ (reverse) and 5′-AAATTTAAGCTTATGATGATTGCTTTCGCACTGCAT-3′ (forward)] and inserted into pCAGGS vector using the CloningEZ Kit (SantoRoga). The gpr126 NT fragment was cloned into pCAGGS vector using Hind III and Cla I restriction sites [primers: 5′-AAATTTAAGCTTATGATGATTGCTTTCGCACTGCAT-3′ (reverse) and 5′-AAATTTAAGCTTATGATGATTGCTTTCGCACTGCAT-3′ (forward)]. This construct was amplified using primers: 5′-AAATTTAAGCTTATGATGATTGCTTTCGCACTGCAT-3′ (forward) and 5′-AAATTTAAGCTTATGATGATTGCTTTCGCACTGCAT-3′ (reverse) and inserted into the signal peptide containing p3XFLAG-CMV-14 vector at the Xho I site using the CloneEZ Kit. A recent paper (30) noted that the reference sequence for the zebrafish gpr126 gene codes for Cys804, but that the wild-type zebrafish strains they analyzed encoded Trp at this position. Our analysis also showed that wild-type fish strains code for Trp at this position, and our constructs all contained Trp at this position. The sequences of all constructs were verified.
Purification of fusion proteins
HEK293 cells were transfected with empty pFUSE vector or expression vectors for our different Gpr126-Fc fusion proteins using Lipofectamine 2000 following the manufacturer’s standard protocol. Cells were incubated at 37°C for 3 days. The conditioned medium was collected and concentrated using Amicon Ultra-15 centrifugation filters. Concentrated conditioned medium was then incubated overnight at 4°C with protein A-agarose to purify fusion proteins. Proteins were then eluted from beads with 1 M NaCl or 0.1 M glycine buffers and dialyzed into Hepes-buffered saline (10 mM Hepes and 150 mM NaCl, pH 7.5). Protein purity was determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver stain, and the concentration was determined by Bradford assay.

Pull-down assays
Purified collagen proteins were obtained from Sigma-Aldrich and were biotinylated using the Pierce EZ-Link Biotinylation Kit. Equal quantities by weight of biotinylated collagen and Gpr126-NT-Fc were mixed and incubated overnight with streptavidin- or protein A-agarose. Beads were washed in Hepes-buffered saline (10 mM Hepes and 150 mM NaCl, pH 7.5) and then eluted by addition of Laemmli sample buffer. Samples were resolved by SDS-PAGE followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, 50 mM NaCl, 20 mM Hepes, and 0.1% Tween 20) for 30 min and then incubated with primary antibody for 1 hour at room temperature. In cases where the primary antibody was not directly coupled to HRP, membranes were washed in blocking buffer and incubated with HRP-conjugated secondary antibody (GE Healthcare) for 30 min. After washing to remove HRP-coupled antibodies, blots were visualized via ECL reagent (Pierce) followed by exposure to film.

cAMP measurements
HEK293 cells were transfected by standard protocol using Lipofectamine 2000 with empty vector or expression plasmid containing various Gpr126 constructs. Cells were treated with collagen for 1 hour unless otherwise indicated. The cells were lysed in 0.1 M HCl, and the intracellular cAMP concentration was measured with an enzyme-linked immunosorbent assay (ELISA)-based kit (EMD Millipore). To determine the EC_{50}, we analyzed the dose-response data with a four-parameter Hill equation using GraphPad Prism software.

SPR-based biosensor analysis
The interaction of Gpr126 variants with type IV collagen was analyzed by SPR using a Biacore 3000 biosensor system (GE Healthcare). Protein dilutions were performed in HBS-N [10 mM Hepes (pH 7.4), 150 mM NaCl] running buffer. Type IV collagen was immobilized onto CM5 biosensor chip by amine coupling chemistry using N-hydroxysuccinimide (NHS) and N′-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). To investigate binding of Gpr126-NT-Fc or CUBPTX-Fc, proteins were diluted in HBS-N buffer and injected over the type IV collagen surface. The experiments were performed at 25°C using a flow rate of 30 µl/min. For each experiment, at least five different concentrations of Gpr126-NT-Fc or CUBPTX-Fc were injected over each experimental and control flow cell. Dissociation was allowed to occur at the same flow rate for 300 s, followed by running buffer alone for 2 min. The surface was regenerated with 0.5 N NaCl for 30 s, followed by 1.0 M NaCl using a flow rate of 100 µl/min, followed by running buffer alone for 2 min to enable stabilization of the baseline. Biosensor experiments were repeated a minimum of two times. All data were corrected for nonspecific binding by subtracting the signal measured in a control cell lacking immobilized ligand. Both data processing and kinetic fitting were performed with Scrubber software, version 2 (BioLogic Software Pty.), or BIAevaluation software 4.1 (Biacore).

Purification and culture of neonatal rat Schwann cells
Rat Schwann cells were acutely isolated from neonatal (P2) rat sciatic nerves by immunopanning, using the method of Cheng and Mudge (66). Briefly, isolated sciatic nerves were digested with collagenase/dispase and then incubated on sequential negative passing plates containing antibodies against CD45 (to deplete macrophages) and Thy 1.1 (to deplete fibroblasts). Schwann cells were then purified to about 99% purity by adherence to an O4 (anti-sulfatide) plate and removed by trypsin. For cAMP assays, acute Schwann cells were plated on poly-D-lysine–coated coverslips in defined, serum-free medium (Dulbecco’s modified Eagle’s medium/Sato containing insulin, sodium pyruvate, l-glutamine, thyroxine, B27, and N-acetylcysteine) in the absence of forskolin or growth factors. Cells were grown overnight before testing.

Fish strains and genotyping
Zebrafish embryos were raised at 28.5°C and staged as described (67). The gpr126<sup>st49</sup> mutation was identified in an ENU (N-ethyl-N-nitosourea)–based screen for mutations that disrupted mbp mRNA expression, following previously described methods for mutagenesis and screening (25). The gpr126<sup>st86</sup> mutation failed to complement the previously described gpr126<sup>st40</sup> allele (26), and sequencing the gpr126 gene from mutants and wild-type siblings identified the lesion. To genotype individual animals in the phenotypic analyses, the gpr126<sup>st86</sup> mutation was scored by a PCR test of genomic DNA samples. We used primers to amplify genomic DNA spanning the lesion (5′-TGTAAAGACTTTGGGTGATT-TTG-3′ and 5′-CATTGAGATATTATCAGCCGTAGC-3′) and digested the products with the restriction enzyme Dpn II. The gpr126<sup>st86</sup> mutation disrupts one of the two Dpn II sites in the wild-type sequence.

Surface biotinylation assay
HEK293 cells transfected with Gpr126-FLAG or Gpr126-NT-FLAG were washed with PBS and suspended in PBS containing 2 mM sulfo-NHS-biotin reagent (Pierce). The biotinylation reaction was incubated for 2 hours on ice. Cells were gently washed in 100 mM glycine solution to quench the reaction and then lysed in buffer with 1% Triton X-100, 300 mM NaCl, and 50 mM tris. The lysate was cleared by high-speed centrifugation. After a portion was set aside to analyze total cell lysates, the remainder of the lysate was incubated with streptavidin-agarose overnight at 4°C to pull down biotinylated proteins. Beads were washed, suspended in sample buffer (5 M urea, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, 50 mM tris), and then resolved by SDS-PAGE followed by transfer to nitrocellulose membranes. Gpr126-FLAG surface expression was assessed by Western blot analysis using a FLAG antibody. As a control cytoplasmic protein, we analyzed the samples in immunoblots with an actin antibody (Thermo Scientific, MS-1295).

Analysis of FLAG constructs
For immunoblots, transfected HEK293 cells transfected with Gpr126-FLAG or Gpr126-NT-FLAG were lysed directly in a urea-based sample buffer (5 M urea, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, 50 mM tris) and then sonicated on ice. For microscopy, HEK293 cells were transfected with empty vector, Gpr126-FLAG, or Gpr126-NT-FLAG, plated onto coverslips, and allowed to attach overnight. Cells were then fixed with 4% paraformaldehyde, washed, and incubated with an antibody against FLAG (1:2000) overnight at 4°C. Cells were washed extensively and then incubated with Alexa Fluor goat anti-mouse 488 secondary (1:500) for 1 hour at room temperature. Slides were viewed using the
Quantitative reverse transcription PCR analysis
Total RNA was extracted from individual embryos using the RNAeasy Micro Kit (Qiagen). Embryos were sorted according to the ear phenotypes described above. Complementary DNA was made using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCR was performed on the Bio-Rad CFX384 Real-Time PCR Detection System with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). gpr126 transcripts were detected using the forward primer 5′-TGTTGTTGAGGTTGATGAC-3′ and reverse primer 5′-CGGTTCCTGGTGAAAGGTG-3′. Values were normalized to the expression of ef1α.

Statistical analysis
Single comparisons for statistical significance were done using an unpaired t test with GraphPad Prism software. Statistical significance was deemed P < 0.05.

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Type IV collagen is an activating ligand for the adhesion G protein–coupled receptor GPR126

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GPR126: An Orphan No More
Cell surface receptors called adhesion G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors (GPCRs) mediate diverse developmental and physiological processes, yet many are "orphan" receptors with unknown ligands. The adhesion GPCR GPR126 is important for the development of Schwann cells, the heart, and the inner ear. Paavola et al. found that type IV collagen, a protein found in the extracellular matrix, is an agonist of GPR126. In cultured cells overexpressing GPR126 and in rodent Schwann cells, type IV collagen stimulated synthesis of cyclic adenosine monophosphate, a downstream effector of GPR126 signaling. The interaction between ligand and receptor was mapped to domains in the N-terminal region of the GPCR. An N-terminally truncated GPR126 construct was constitutively active, suggesting that this region of the receptor represses signaling in a way that is relieved by ligand binding. Thus, these findings implicate type IV collagen as a ligand of GPR126 in developmental signaling.