Structural basis for adhesion G protein-coupled receptor Gpr126 function

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Structural basis for adhesion G protein-coupled receptor Gpr126 function

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Many drugs target the extracellular regions (ECRs) of cell-surface receptors. The large and alternatively-spliced ECRs of adhesion G protein-coupled receptors (aGPCRs) have key functions in diverse biological processes including neurodevelopment, embryogenesis, and tumorigenesis. However, their structures and mechanisms of action remain unclear, hampering drug development. The aGPCR Gpr126/Adgrg6 regulates Schwann cell myelination, ear canal formation, and heart development; and GPR126 mutations cause myelination defects in human. Here, we determine the structure of the complete zebrafish Gpr126 ECR and reveal five domains including a previously unknown domain. Strikingly, the Gpr126 ECR adopts a closed conformation that is stabilized by an alternatively spliced linker and a conserved calcium-binding site. Alternative splicing regulates ECR conformation and receptor signaling, while mutagenesis of the calcium-binding site abolishes Gpr126 function in vivo. These results demonstrate that Gpr126 ECR utilizes a multi-faceted dynamic approach to regulate receptor function and provide relevant insights for ECR-targeted drug design.
Multicellular organisms rely on cellular communication to carry out critical biological processes, and numerous cell-surface receptors utilize their extracellular regions (ECRs) to modulate these cellular-adhesion and signaling events. For example, the ECRs of integrins, epidermal growth factor receptor (EGFR), and several G protein-coupled receptors (GPCRs) change conformation upon ligand binding, which propagates signals across the membrane. Targeting the essential ECRs of receptors with antibody-like drugs to trap the ECRs in order to treat inflammatory bowel diseases. Remarkably, earlier this year, the migraine preventive drug eumunab, which blocks ligand binding to the ECR of calcitonin receptor-like receptor, became the first antibody drug against a GPCR to be approved by the Food and Drug Administration. Like all GPCRs, aGPCRs have canonical signaling seven-transmembrane (7TM) domains. However, unlike most other GPCRs, aGPCRs have large ECRs, which can extend up to almost 6000 amino acids (aa) and consist of various adhesion domains that mediate cell–cell and cell–matrix interactions. In addition, during biosynthesis, aGPCRs are uniquely autotropoless within a conserved GPCR Autotrotolesis Inducing (GAIN) domain of the ECR that is juxtaposed to the 7TM, resulting in a fracted receptor that nevertheless remains tightly associated at the cell surface.

Although their protein architectures remain largely unknown, functional studies have shown that aGPCR ECRs can regulate receptor function and that antibody-like synthetic proteins that target the ECRs can modulate downstream signaling. A current model for aGPCR regulation suggests that transient interactions between the ECR and 7TM directly regulate receptor function, a feature that is likely true for other aGPCRs, and that will form the basis for further investigations in the efforts to drug aGPCRs.

Gpr126/Adgrg6 is one of the better studied aGPCRs and is essential for Schwann cell (SC) myelination and other functions. In vertebrate peripheral nervous system (PNS) development, the myelin sheath surrounding axons is formed by SCs to modulate these cellular-adhesion and signaling events. A second feature that also mediates the closed conformation is a newly identified calcium-binding site at the tip of the ECR. Strikingly, zebras with point mutations at this site have both myelination defects and malformed ears, demonstrating the critical role of the ECR in Gpr126 function in vivo. These results altogether show that the ECR of Gpr126 has multifaceted roles in regulating receptor function, a feature that is likely true for other aGPCRs, and that will form the basis for further investigations in the efforts to drug aGPCRs.

Results

Structure of the full-length ECR of Gpr126. To determine the structure of the ECR of Gpr126, the full-length ECR (−ss) from zebras with Gpr126 (T39-S837) was expressed and purified from insect cells using the baculovirus expression system. Zebras with Gpr126 (Fig. 1a) has high sequence identity (47%) to its human homolog but its ECR has a fewer number of N-linked glycosylation sites (15 predicted in zebra and 22 in human) and no furin-cleavage site (Supplementary Fig. 1A, B), and thus yields a more homogeneous sample (Supplementary Fig. 1B, C). Crystals of the ECR from both native and selenomethionine (SeMet)-labeled zebras with Gpr126 ECR (−ss) were obtained and diffraction 2.4 Å (Supplementary Fig. 1D), and the structure was determined by SeMet single-wavelength anomalous diffraction (SAD) phasing (Supplementary Table 1).
The structure, with overall dimensions of $110 \times 80 \times 35 \text{ Å}$, revealed the presence of five domains (Fig. 1b, c), of which only four were identified previously. The N-terminal region of the protein is composed of the CUB domain followed very closely by the PTX domain. The 150 aa unknown region after the PTX domain was revealed to be a 22 aa linker that is partially disordered, the 23 aa alternatively spliced region (not present in the crystal structure construct), and a structured domain which spans 105 aa and was identified as a SEA domain through the Dali server\textsuperscript{55}. The Gpr126 SEA domain adopts a ferredoxin-like alpha/beta sandwich fold, common to SEA domains from other proteins. Interestingly, analysis of the structure as well as sequence alignments between zebrafish and human showed that furin cleavage in humans would occur in the SEA domain (Supplementary Fig. 1A). Finally, the SEA domain is followed by the HormR and GAIN domains, the latter of which is autoproteolysed as expected (Supplementary Fig. 1E). The HormR and GAIN domain structures are similar to previously-solved HormR + GAIN domain structures from other aGPCRs\textsuperscript{9,20}, with the exception of the relative orientation between HormR and GAIN. There is a 90° rotation of the HormR domain with respect to the GAIN domain (Supplementary Fig. 1F) in Gpr126 compared to previously-solved HormR + GAIN structures from rLphn1 and hBAI3\textsuperscript{20}. In addition, Gpr126 was observed to have at least ten sites of glycosylation throughout all domains of the ECR except the PTX domain (Fig. 1b).

Gpr126 (−ss) ECR adopts a closed conformation. Unexpectedly, the structure revealed a compact, closed conformation where the most N-terminal CUB domain interacts with the more C-terminal HormR and GAIN domains (Fig. 1b). To ensure that this conformation is not a crystallization artifact, we utilized both negative-stain electron microscopy (EM) and small-angle X-ray scattering (SAXS) to confirm that the closed conformation is observed for Gpr126 in solution. Negative-stain 2D class averages of Gpr126 (−ss) ECR showed a V-shaped protein architecture (Fig. 1d). The individual domains in the 2D class averages were assigned according to size and are consistent with the closed architecture of the crystal structure. In addition, we measured the radius of gyration ($R_g$) of the ECR using SAXS to confirm that the closed conformation exists in solution. The observed $R_g$ (41.1 ± 0.1 Å) is...
**Fig. 2 Closed conformation of Gpr126 is mediated by CUB-HormR-linker interactions.**

a. Structure of the full ECR of (-ss) Gpr126. b. Close-up view of the CUB-HormR interface. Residues at the interface are shown as sticks. The calcium ion is shown as a bright green sphere. c. Close-up view of the calcium-coordination site within CUB domain. The water molecule is shown as a blue sphere. The residues are shown as sticks. CUB residues are colored dark blue and HormR residue is colored yellow. Residue labels are colored according to their roles in CUB-HormR interaction: red (E89, D97, D134) represents calcium coordination by side-chain residue, blue (S136, V137) represents calcium coordination by main-chain carbonyl group, purple (F135) represents a hydrophobic residue in CUB-HormR interface, and orange (Y61) represents a residue that stabilizes calcium-coordinating residue D97. Calcium coordination is shown as bright green dashed lines. CUB-HormR interaction is shown as yellow dashed lines. The interaction between Y61 and D97 is shown as a magenta dashed line.

d. Sequence alignment of partial Gpr126 CUB domain from various species, highlighting important conserved residues: calcium-coordinating residues by side-chain group (red), calcium-coordinating residues by main-chain carbonyl (blue), a tyrosine residue that stabilizes a calcium-coordinating residue (orange), and a hydrophobic phenylalanine residue in the CUB-HormR interface (purple).

e. Close-up view of the disulfide-stabilized loop inserted between CUB and HormR domains. The disulfide bond is colored bright orange and is indicated by an arrow. The dashed line represents disordered residues in the linker region.

Consistent with the calculated $R_g$ of the closed-conformation crystal structure model (42.6 Å) and inconsistent with that of an extended model of Gpr126 ECR in which the CUB domain points away, rather than toward, the center of the molecule ($R_g = 52.2$ Å) (Supplementary Fig. 1G). Taken together, these results: show that Gpr126 ECR is in a closed conformation in solution, demonstrate that this conformation is not an artifact of crystal-packing contacts, and suggest that this closed conformation may play an important role in Gpr126 function.

As the closed conformation of Gpr126 (-ss) ECR was shown to exist both in solution and in the crystal lattice, we next wanted to explore the interactions that contribute to this protein architecture. Close examination of the crystal structure revealed two interaction sites that mediate the closed conformation, the first of which is a direct interaction between domains that are at opposite ends of the ECR and the second is an indirect interaction formed between two domains through a loop that holds them together (Fig. 2a).

First, a direct interaction exists at the tip of the CUB domain (close to the N-terminus), which points inward towards the center of the molecule and lies in the interface between GAIN and HormR. Residues in the HormR domain (H516, F533, P534, Y535) interact with each other through pi-pi stacking (sandwich), promoting interaction with F135 on the CUB domain through additional (T-shaped) pi-pi stacking to stabilize the CUB-HormR interaction (Fig. 2b).

Surprisingly, examination of the 2Fo-Fc electron density map showed that there is density within the CUB domain at this interface that does not belong to any amino acid residue (Supplementary Fig. 2A). This density is coordinated by the side-chain groups of E89, D97 (bidentate) and D134, main-chain carbonyl groups of S136 and V137, as well as a water molecule for a complex with coordination number 7 in a pentagonal bipyramid geometry (Fig. 2c). The geometry and distances between the density and the coordinating residues in Gpr126 are consistent with calcium coordination. Several CUB domains from extracellular proteins are reported to coordinate calcium, including Gpr126, and some have been discovered to use this coordination to mediate ligand binding (Supplementary Fig. 2B). For example, the C1s protein uses its CUB calcium-binding site to bind to ligand C1q and initiate the classical pathway of complement activation, and the Lujo virus recognizes a calcium-binding site on the CUB domain of the neurophilin-2 receptor in order to gain cell entry. The calcium-coordinating residues are all conserved in the Gpr126 CUB domain (among GPR126 proteins from various species (Fig. 2d) as well as among calcium-binding CUB domains from other proteins (Supplementary Fig. 2C)), suggesting that the density is indeed calcium. Importantly, the calcium coordination aligns the coordinating residues E89 and D134 on the surface of the CUB domain such that they can interact with K356 on the HormR domain (Fig. 2c), contributing to the closed conformation.

In addition to the direct CUB-HormR interaction, a second interaction site is formed by a disulfide-stabilized loop, which provides a bridge between the CUB and HormR domains. Although 13 (C355-A367) of the 22 aa (C355-P376) in the linker...
region are disordered in the structure, the rest were able to be resolved and they form a small loop stabilized by a disulphide bond between C369 and C375 (Fig. 2e). This loop is located directly N-terminal to the SEA domain and is inserted between the CUB and HormR domains, effectively bridging the two domains and likely contributing to the stabilization of the closed conformation. The cysteines that form the disulphide bond are conserved among all except four of the 94 species analyzed in this study (Supplementary Fig. 2D and Supplementary Data 1), suggesting that this disulphide bond plays an important role in Gpr126 function. The five residues (ASGLG) flanked by the cysteines are small and flexible, accommodating the formation of the disulphide loop as well as insertion into the small pocket between CUB and HormR.

Alternative splicing modulates Gpr126 ECR conformation. Gpr126 is alternatively spliced, producing several isoforms that may modulate protein function. Skipping of exon 6 results in deletion of 23 aa in zebrafish (28 aa in human) and is of particular interest because these amino acids reside in the previously unknown region of Gpr126 ECR. The 23 aa region is rich in serine/threonine residues (10 out of 23) and contains a predicted N-linked glycosylation site, which suggests that this region may be a highly O- and N-link glycosylated stalk. From analysis of the crystal structure (−ss isofom, in which the 23 aa are deleted), we determined that the splice site is directly between the regions encoding the disulfide-stabilized loop and the SEA domain (Fig. 3a). Because the disulfide-stabilized loop makes contacts that are important for the closed conformation of Gpr126 ECR (−ss) (Fig. 2e), we hypothesized that the (+ss) isoform would disrupt the closed conformation and have a different, more open conformation.

To test whether Gpr126 ECR (+ss) and (−ss) have different conformations, the two proteins were purified and analyzed using negative-stain EM. Single particles were classified into 2D class averages and the class averages were further categorized into groups to facilitate interpretation of different conformations. The class averages for the (−ss) isoform, categorized into five main orientations (Fig. 3b), were consistent with the closed conformation of the crystal structure (Fig. 1b). However, the class averages for the (+ss) isoform (Fig. 3c) showed a diverse population of ECR molecules, as they contain additional more open-like conformations (group vi, 21% of particles, Fig. 3d) as well as closed conformations that were observed in the (−ss) isoform (Fig. 3c, d). Furthermore, individual (+ss) particles showed the presence of open conformations (Fig. 3e), including a fully extended conformation, which could not be classified into a distinct class average during image processing. These results are consistent with our hypothesis that the (+ss) ECR conformation is different from that of (−ss) and suggest that the addition of 23 aa extends the linker in (+ss), likely disrupting the indirect and direct CUB-HormR interactions and preventing the stable closed conformation that is observed in (−ss) (Fig. 3f).

The negative-stain EM data are consistent with SAXS experiments showing that the Rg of zebrafish Gpr126 ECR (+ss) is larger than that of (−ss) with a more dramatic change in Rg observed between the human GPR126 isoforms (Supplementary Fig. 3A–F and Supplementary Table 2). Size-exclusion chromatography elution profiles for both zebrafish and human constructs also showed that (+ss) elutes earlier compared to (−ss), indicative of a larger size and different shape (Supplementary Fig. 3G, H).

Alternative splicing modulates Gpr126 receptor signaling. To determine whether the two isoforms also exhibit different levels of signaling, receptor activity was measured for both isoforms using a G protein signaling assay. Human GPR126 has been shown previously to couple to and activate Gαs, leading to production of cAMP. Therefore, we used a cAMP signaling assay in which HEK293 cells were co-transfected with a full-length zebrafish Gpr126 construct and a reporter luciferase that emits light upon binding to cAMP. Cell-surface expression levels of the constructs were quantified by flow cytometry analysis of cells stained by antibodies against N-terminal FLAG-tags (Fig. 4a and Supplementary Fig. 4A, B, C), and basal signaling results (Fig. 4b) were normalized to expression level (Fig. 4c).

Cells transfected with either (−ss) or (+ss) Gpr126 had higher cAMP levels compared to cells transfected with an empty vector (EV) (Fig. 4c), demonstrating that basal activity of Gpr126 can be detected in this assay. As a positive control, a synthetic peptide agonist that targets the 7TM activated zebrafish Gpr126 and human GPR126 signaling to a level consistent with similar, previously-published experiments on human GPR126 (Supplementary Fig. 4D) and did not activate signaling in EV-transfected cells.

However, the closed-conformation (−ss) Gpr126 signaled significantly less compared to the more dynamic (+ss) Gpr126 (Fig. 4c), and this result was consistent between both zebrafish and human constructs (Supplementary Fig. 4C and Fig. 4d–f). This suggests that the additional amino acids in the linker region of the ECR as a result of alternative splicing plays a role in modulating the activity of Gpr126 and that the ECR of Gpr126 is coupled to receptor signaling. Taken together with the negative-stain EM results, the (−ss) and (+ss) Gpr126 isoforms are distinct in terms of ECR conformation dynamics, as well as G protein signaling activity.

In addition, we mutated calcium-binding site residues D134A/F135A in the (−ss) isoform, which we predicted would disrupt the closed conformation. Using negative-stain EM, we observed open ECR conformations for this construct (Supplementary Fig. 4E–G), similar to the wild-type (+ss) isoform (Fig. 3c and Supplementary Fig. 4H). The calcium-binding site mutation did not increase or decrease the cAMP signaling for the (−ss) Gpr126 isoform, which suggests that the ECR conformation is not solely responsible for regulation of receptor signaling. However, the same mutation in the (+ss) isoform resulted in lower cAMP levels compared to wild-type (+ss) (Supplementary Fig. 4I). Cell-surface expression levels of these mutant Gpr126 constructs in HEK293 cells were similar or higher than wild-type constructs, excluding the possibility that lower signaling was due to improper protein folding or trafficking (Supplementary Fig. 4I). Altogether, these results might be explained by a complex, rather than a simple and straightforward, model of regulation for receptor signaling and suggest a possible functional role for the calcium-binding site.

Calcium-binding site is critical for PNS myelination in vivo. Functional sites on proteins are usually highly evolutionarily conserved. We used the ConSurf server to perform surface conservation analyses on a diverse set of 94 Gpr126 protein sequences. The conservation score for each residue was mapped onto the Gpr126 ECR structure (Supplementary Fig. 5A), which revealed that the most conserved domain in the ECR is the CUB domain. Importantly, the calcium-binding site is absolutely the most highly conserved patch within the CUB domain and within the entire Gpr126 ECR (Fig. 5a). The calcium-binding site is universally conserved among all species analyzed, which suggests that the calcium-binding site has an essential role in Gpr126 function.

We next wanted to test whether the residues in the calcium-binding site are important for Gpr126 function in vivo. Gpr126 has previously been shown to regulate both PNS myelination and
ear development in zebrafish through elevation of cAMP\textsuperscript{35,36}. Zebrafish gpr126 mutations that impair G protein signaling result in abolished myelination of the peripheral axons by SC and cause “puffy” ears\textsuperscript{28,32,35,36,63}. GPR126 has been shown to have a role in heart development in mouse\textsuperscript{37}, supported by additional studies in zebrafish\textsuperscript{28,30,63}. Gpr126 activity in zebrafish can be readily measured by analyzing the expression of myelin basic protein (mbp), which encodes a major structural component of the myelin sheath and is essential for PNS myelination, and by assessing ear and heart morphologies of the fish. To determine whether the calcium-binding site is important for these functions, two amino acids in the site, D134 and F135, were targeted and mutated to alanines using CRISPR/Cas9-mediated homologous recombination. D134 directly coordinates the calcium ion and F135 is an adjacent hydrophobic residue which forms one arm of the calcium-binding pocket (Figs. 2c, 5a). As a result, the mutant zebrafish, gpr126\textsuperscript{stl464}, harbor D134A and F135A mutations (Fig. 5b, Supplementary Fig. 5B). These mutations created a BstUI restriction enzyme site, which was used to genotype individual zebrafish (Fig. 5c). Expression of gpr126 is unaffected in gpr126\textsuperscript{stl464} mutants (Supplementary Fig. 5C, D). Strikingly, compared to wild-type siblings, the gpr126\textsuperscript{stl464}-mutant zebrafish developed the puffy ears (Fig.5d, e) that are indicative of a defect in Gpr126-mediated G protein signaling, though they do not appear to have heart defects (Supplementary Fig. 5E-T). In addition to the ear phenotype, mutant zebrafish did not express mbp, indicative of failed PNS myelination (Fig. 5f, g, Supplementary Fig. 5U, V). These results show that D134 and F135 in the calcium-binding pocket of Gpr126 are essential for ear and SC development in vivo.
Identification of a proteolytic SEA domain in human GPR126.

As mentioned earlier, the previously unknown region in the Gpr126 ECR contains a structured domain, which we revealed to be a SEA domain (Fig. 6a). Gpr126 SEA superimposes well over known SEA domains from Mucin-1 and Notch-264,65, which are cleaved (via autoproteolysis and furin, respectively), both in the same loop between beta-strand 2 and beta-strand 3 (Fig. 6b, c).

Although the GPR126 furin-cleavage site is conserved in many mammals and birds (Supplementary Data 1, Supplementary Fig. 1A), with a consensus sequence of (R/K)-X-K-R↓, it is not conserved in zebrafish Gpr126. Using sequence alignments (Fig. 6d) and homology modeling, we mapped the furin-cleavage site in human GPR126 (Fig. 6e, Supplementary Fig. 6A) to the same loop that is cleaved in Mucin-1 and Notch-2, suggesting that SEA domain cleavage plays similar roles in each of these proteins. Consistent with a previous study51, R468A mutations...
abolish furin cleavage in both human GPR126 (−ss) and (+ss) isoforms (Supplementary Fig. 6B, C). In addition, these mutant GPR126 constructs were transfected into HEK293 cells and were detected on the cell surface (Supplementary Fig. 6D), and therefore, the importance of furin cleavage is likely not primarily important for proper expression and trafficking.

To our knowledge, SEA and GAIN are the only known protein domains that are proteolyzed and remain associated even after proteolysis. In proteins like Mucins and Notch, the cleaved SEA domain remains intact and shear forces likely unfold the domain and separate the protein into two fragments. The Gpr126 SEA domain shows several noncovalent interdomain interactions, particularly between all four of the beta-strands that form a beta-sheet (Fig. 6f). The separation of the human GPR126 furin-cleaved SEA domain into two fragments does not readily occur immediately following cleavage as the cleaved protein resists separation when purified by size-exclusion chromatography (Supplementary Fig. 6B), similar to the aforementioned SEA domains as well as to GAIN domain autoproteolysis. Instead, the two fragments likely stay associated noncovalently until a disruptive event, such as ligand binding and mechanical force, unfolds the SEA domain and leads to separation or shedding of the region N-terminal to the furin-cleavage site (CUB, PTX, linker, half of SEA) and the C-terminal region (half of SEA, HormR, GAIN, 7TM).

**Discussion**

aGPCRs make up the second largest family of GPCRs with 32 members in humans and are essential for numerous biological processes such as synapse formation, cortex development, neutrophil activation, angiogenesis, embryogenesis, and many more. Recent studies have shown that the ECRs of aGPCRs play important roles in these functions; however, the relative lack of information about the structures of ECRs and their mechanisms of activation hampers further studies toward drugging these receptors. Here we show that the large ECR of Gpr126, an aGPCR with critical functions in PNS myelination, ear development, and heart development, adopts an unexpected closed conformation where the most N-terminal CUB domain interacts with the more C-terminal HormR domain. The structure of the Gpr126 ECR revealed that the closed conformation is mediated through a calcium-binding site as well as a disulfide-stabilized loop. Interestingly, the residues involved in these intramolecular interactions are highly conserved among Gpr126 sequences, including that of zebrafish, raising questions about their role in Gpr126 function.

Alternative splicing is a mechanism to increase the functional diversity of metazoan genomes and has been repeatedly demonstrated to play a role in the regulation of brain function. For example, alternative splicing contributes to the functional diversification of DSCAMs, protocadherins, channel proteins, neurexins, and neuroligins. It is also proposed that alternative splicing may cause a large conformational change in the ECR of the synaptic protein teneurin, since alternative splicing allows the protein to act as a switch in regulating ligand binding despite the ligand-binding site being distant from the seven aa alternatively spliced site. Since gpr126 is alternatively spliced in the region encoding the ECR, we examined the functional differences between isoforms. Our negative-stain EM and SAXS results suggest that alternative splicing between the regions encoding the PTX and SEA domains in gpr126 perturbs the closed conformation and generates a population of ECR conformations that range from closed to extended (Fig. 7a). Several of the inserted residues resulting from alternative splicing are predicted to be sites of glycosylation. These glycosylation sites as well as the state of the other glycosylation sites may contribute to the change in ECR conformation. Our signaling assay results also show that alternative splicing leads to changes in basal receptor activity, which suggests that the architecture and conformation of aGPCR ECRs play more important roles in their functions than previously thought. However, the signaling assay results showing that the change of Gpr126 ECR conformation is not solely responsible for changes in signaling may be confusing and contradictory. Rather, a more complex model that combines changes in ECR conformation with exposure of potential functional sites due to these changes may be key for alternative splicing-mediated regulation.

Importantly, we identified the calcium-binding site in Gpr126 as a potential functional site. Our in vivo results showed that zebrafish carrying two point-mutations in the calcium-binding site have defective SC and ear development, suggesting that the calcium-binding site is essential for the in vivo functions of Gpr126 (Fig. 7b). Since a subset of CUB domains from other

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**Fig. 5 The calcium-binding site is required for Gpr126 function in vivo.** a Surface conservation analysis (green, variable; purple, conserved) of CUB domain. The calcium-binding site is circled in magenta. D134 and F135 are indicated by arrows. D134 and F135 were both mutated to alanines through homologous recombination of a 150 bp ssODN containing a 5 bp mutation (red nucleotides). b Genotyping assay for the gpr126stl464 lesion. The 5 bp mutation introduces a BstUI restriction enzyme binding site. The 5 bp mutation introduces a BstUI restriction enzyme binding site.
proteins coordinate calcium in order to mediate ligand-binding\textsuperscript{57}, one possibility for the critical function of the calcium-binding site in Gpr126 may be to act as a ligand-binding site as well, although future experiments will need to be performed to validate this hypothesis.

The structure also revealed the presence of a SEA domain. In human and other species, a furin-cleavage site is mapped to this domain but this cleavage site is not conserved in zebrafish. Therefore, the function of the furin-cleavage may play a role in GPR126 that is not conserved in zebrafish. Cleaved SEA domains from other proteins have been shown to stay intact until a force is applied and pulls apart the fragments\textsuperscript{67,68}. Similarly, GPR126 may regulate its activity by furin-dependent shedding in addition to the established GAIN-autoproteolysis-dependent shedding. Moreover, the released extracellular fragments may act as diffusible ligands and bind to other cell-surface receptors, but further studies need to be done to test this model. Other aGPCRs that have SEA domains in their ECRs include ADGRF1/GPR110 and ADGRF5/GPR116\textsuperscript{74,75}. Although these SEA domains are not cleaved by furin, they do contain the GSVVV (or GSIVA) motif that leads to autoproteolytic cleavage in the same loop (between beta-strand 2 and beta-strand 3) that is cleaved by furin in GPR126. Therefore, SEA domain cleavage, whether by autoproteolysis or by furin, is a common feature in several aGPCRs and may have similar roles in regulating receptor function.

Taken together, our results suggest that Gpr126 is a complex protein that makes use of its many domains to regulate its function. In addition to the autoproteolysis-dependent activation mechanism (Supplementary Fig. 7A), Gpr126 uses other mechanisms to regulate its function including modulation of the ECR conformation. In the closed conformation, Gpr126 signals less compared to when the ECR is in a more dynamic, open conformation, which may be regulated by alternative splicing (Fig. 7a). Alternative splicing which deletes the CUB domain\textsuperscript{30}
may also regulate receptor function (Supplementary Fig. 7B). Mutation of the calcium-binding site leads to signaling defects in vitro and to ear and PNS defects in vivo (Fig. 7b)). In addition, furin cleavage may allow GPR126 another mode of activation that is common to other receptors and adhesion GPCRs.

The Gpr126 closed conformation and hidden calcium-binding site is conceptually similar to EGFR. EGFR is in a closed, compact inactive conformation until ligand binding leads to a conformational change that extends the protein and reveals a hidden functional site that is important for its activation16,76. Because this mechanism is key for drugging EGFR, the conceptual similarity provides an opportunity to also drug Gpr126. Drugs that alter the ECR conformation of Gpr126 or block functional sites, such as the calcium-binding site, may be useful for treating Gpr126-associated diseases. The ECRs of other aGPCRs are major players in mediating receptor functions as well. For example, using its ECR, ADGRA2/GPR124 regulates isoform-specific Wnt signaling77–80, the C. elegans ADGRL1/LAT-1 controls cell division planes during embryogenesis, and ADGRB1/BAI1 and ADGRL3/Lphn3 mediate synapse formation through interaction with other cell-surface proteins81–84. Thus, the ECRs of other aGPCR family members are also promising drug targets to treat numerous diseases once mechanistic details about their regulatory functions are understood.

**Methods**

**Cloning and purification of Gpr126/GPR126 from insect cells.** The ECRs (residues T39–S837) of zebrafish Gpr126 and ECRs (residues C38–A853) of human GPR126, along with C-terminal 8His-tags, were cloned into the pAcGP67α vector. The following primers were used for amplification of zebrafish Gpr126: F: 5'-CGCAATTGTGGTTGCGAAGAAAGACCAGCTGCAATGGGTG-3' and R: 5'-GAATTCGAAAGTTACCGCTGTTAGTGCTGAGTCGATGATG-3'. The following primers were used for amplification of human GPR126: F: 5'-TCTGCTTTTGCGCGAGCACCAGGCATGGTTGCGAAGAAAGACCAGCTGCAATGGGTG-3' and R: 5'-GGCTGCTTGCTCCCAAAGAGCGCTCCACCTAACTCTGCC-3'. All proteins were expressed using the baculovirus method. Sf9 cells (Thermo Fisher, 12659017) were co-transfected with the constructed plasmid and linearized baculovirus DNA (Expression Systems, 91-002) using Cellfectin II (Thermo Fisher, 10362101). Baculovirus was amplified in Sf9 cells in SF-900 III medium supplemented with 10% FBS (Sigma–Aldrich, F9294). High Five cells (Thermo Fisher, R850502) at a density of 2.0 × 10^6 cells mL^-1 in Insect-XPRESS medium (Lonza, 12-730Q) were infected with high-titer baculovirus and incubated for 72 h at 27 °C. All subsequent steps are conducted at 25 °C. The cells were pelleted at 900 × g for 15 min and the conditioned medium containing the secreted glycosylated proteins were collected. To the medium were added HBS buffer (10 mM HEPES pH 7.2, 150 mM NaCl) containing 20 mM EDTA. Puriﬁcation of Gpr126 was performed in Sf9 cells in SF-900 III medium supplemented with 10% FBS (Sigma–Aldrich, P9294). High Five cells (Thermo Fisher, R85502) at a density of 2.0 × 10^6 cells mL^-1 in Insect-XPRESS medium (Lonza, 12-730Q) were infected with high-titer baculovirus and incubated for 72 h at 27 °C. All subsequent steps are conducted at 25 °C. The cells were pelleted at 900 × g for 15 min and the conditioned medium containing the secreted glycosylated proteins were collected. To the medium were added HBS buffer (10 mM HEPES pH 7.2, 150 mM NaCl) containing 20 mM imidazole. Protein purity was eluted with HBS buffer containing 200 mM imidazole and run on size-exclusion chromatography (Superdex 200 10/300 GL; GE Healthcare) in HBS buffer.

Selenomethione-labeled Gpr126 (−ss) ECR was expressed as previously described85. Briefly, High Five cells in Insect-XPRESS medium were adapted to ESF921 medium (Expression Systems, 96-001-01). The cells were subsequently centrifuged at 100 × g for 15 min and resuspended in ESF921 methionine-free medium (Expression Systems, 96-200). The cells were expanded in the same medium and then infected at a density of 2.0 × 10^6 cells mL^-1 with high-titer baculovirus. At 10 h post-infection, 100 mg Seleno-L-methionine (Sigma–Aldrich, S3132) was added to each liter of cell culture. At 36 h post-infection, another 150 mg Seleno-L-methionine was added to each liter of cell culture. The cells were harvested 72 h post-infection and the purification process was the same as described above.

**X-ray crystallography.** Purified Gpr126 (−ss) ECR (both native and SeMet-labeled) was crystallized at 3 mg mL^-1 in 50 mM potassium dihydrogen phosphate, 29% (w/v) PEG 8000. Both native and SeMet-labeled datasets were collected to 2.4 Å at the Advanced Photon Source at Argonne National Laboratory (beamline 23-ID-D). The datasets were processed with HKL2000 and an initial model was
determined by SAD phasing using Cryo2 and CCP4. Refinement was performed with both REFMAC5 (CCP4) and phenix.refine (PHENIX).

**Negative-electron microscopy.** Uranyl formate (0.75%) solution was freshly prepared by adding 5 ml boiling water to 37.5 mg uranyl formate (Electron Microscopy Sciences, 22450). After stirring for 5 min in the dark, 10 μl 5 M NaOH was added and stirred for an additional 5 min. The solution was syringe filtered (Millipore, SLGV033RS) and stored in the dark. Purified Gpr126 (ss) and (ss), and Δ314A/Δ315A PCR constructs were diluted to 5 μg ml⁻¹ and applied to glow-discharged EM grids (Electron Microscopy Sciences, CF-400-Cu) using a conventional negative-stain protocol⁷⁶. To the grid was applied 2 μl diluted protein for 30 sec. The protein was blotted off with filter paper (Sigma–Aldrich, WHA01110), and then the grid was touched to a 25 μl drop of distilled, filtered water. The water was blotted off, and the grid was touched to a second 25 μl drop of distilled, filtered water. The grid was then touched to a 25 μl drop of 10% uranyl formate for 30 sec and blotted off. The grid was air-dried for 30 sec. The sample was imaged on a Tecnai G2 F30 operated at 300 kV. Gpr126 was visualized in 6565 particles, and Δ314A/Δ315A (3196 particles) were processed using EMAN²⁹.

**Small-angle X-ray scattering.** SAXS measurements were performed at the Advanced Photon Source at Argonne National Laboratory (beamline 18-ID) with an in-line SEC columns (Superdex 200 5–650 10–300) equilibrated with 20 mM HEPES, pH 7.4, and 150 mM NaCl. Data were analyzed using autotag and datognom using the commands “autotag”—mining 0.55–0.85 maxtag 1.1—and “datognom” to create a high-quality output file, respectively, where “1” is the 5'-end. Autotag was performed using the following parameters: F: 5'-GGCTGACTACAAAGACGATGACGACAAGCTTTGCGCAAACTGTAGGGT3'- and R: 5'-CCCTGGCCAGGCTCTGCTATGAGGGTTGTTTGGGTAATTTCCCACTTACTTCCAC-3' using Crysol version 2.8⁹.

**cAMP signaling assay.** Full-length wild-type and mutant Gpr126/GPR126 constructs were cloned into pCMV6. All constructs include N-terminal FLAG-tags for measuring cell-surface expression levels. The following primers were used for amplification of zebrafish Gpr126: F: 5’-GGCTGACTACAAAGACGATGACGACAAGCTTTGCGCAAACTGTAGGGT3’- and R: 5’-CCCTGGCCAGGCTCTGCTATGAGGGTTGTTTGGGTAATTTCCCACTTACTTCCAC-3’. Design of ssODN and microinjections. One-cell stage wild-type embryos were injected with either 2 or 3 nl of a solution containing 132 ng µl⁻¹ of gRNA, -148 ng µl⁻¹ of Cas9 mRNA (obtained from the Hope Center Transgenic Core at Washington University in St. Louis), and 60 ng µl⁻¹ of the ssODN. The 150 bp ssODN was designed from ssODN containing a 5 bp mutation (uppercase): F: 5’-GGATGGGTTCGTTTGTCACGCGCTATTCGAGTTTTTCCAC-3’, and R: 5’-GGACGGCCTGTCGGTTGCTTCCCTGATACCCAGCGC-3’. The PCR product was then cut with an enzyme that is not present in the reference sequence. The 150 bp ssODN was ordered from IDT and contained a 5 bp mutation (uppercase): F: 5’-GATGGGTTCGTTTGTCACGCGCTATTCGAGTTTTTCCAC-3’, and R: 5’-GGACGGCCTGTCGGTTGCTTCCCTGATACCCAGCGC-3’.

**Genotyping.** To identify carriers of the gpr126ΔΔ allele, several primers were used to amplify the 381 base pair (bp) locus of interest: F: 5’-GGTGGTGCAAGAACCGCAGC-3’ and R: 5’-TTCAAGCCTTCACGACTATTTC-3’. After amplification, the PCR product was digested with either DrdI (NEB) or BstUI (NEB) at 60 °C, and then run on a 3% agarose gel. The mutation both disrupts a DrdI binding site and introduces a BstUI binding site. DrdI cleaves wild-type PCR product into 275 and 105 bp products, and the mutant product is 380 bp. BstUI cleaves mutant PCR product into 274 and 106 bp products, and the wild-type product is 380 bp. We recommend using BstUI for genotyping. Any larvae identified with the puffy ear phenotype were always genotyped as gpr126ΔΔ homozygous mutant (n = 20/20).

**Guided RNA synthesis.** Potential gRNA templates were generated by CHOPchop (http://chopchop.cbu.uib.no/). The chosen forward and reverse oligonucleotides, 20 basepairs upstream of the PAM sequence, were ordered with an extra nucleotide at the 5'-end to permit cloning into the pDR274 vector⁷⁶. The oligonucleotide forward sequence used was: 5’- tag GCC TTT AGT GTC CAA AAG AA TTT GCT CAC GC-3’ and oligonucleotide reverse sequence used was: 5’- aac cTT CTT TGT GAG ACT AAA GT-3’; 2 μM of each oligonucleotide was mixed in annealing buffer (10 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA) and incubated at 90°C for 5 min, then cooled slowly to 25 °C over a 45 min time interval. The pDR274 vector was linearized with BsaI and oligonucleotides were ligated into the vector with T4 ligase (NEB) for 10 min at room temperature. The ligation reaction was transformed into competent cells and then plated on kanamycin LB plates. Selected colonies were grown, miniprepped (Zyppy Plasmid Kits, Zymo Research), and sequenced. The gRNA DNA sequence was then PCR amplified from 50 ng µl⁻¹ of the plasmid with Phusion (NEB) and the following primers: F: 5’-GTTGGAAACCTTCTACTGAT GCC-3’ and R: 5’-AAAAGACCGACTCTGGTG-3’. The PCR product was then cut with enzyme that is not present in the reference sequence. The enzyme nucleotide was not integrated into the stabΔΔ4 mutant. At 1 dpf, embryos were genotyped for disruption of the wild-type DrdI binding site and screened for the characteristic gpr126 puffy ear mutant phenotype. Mutations that were successfully screened the F1 offspring were then cultured to the largest for analysis. Mutant bands were gel extracted (Qiagen Gel Extraction Kit) and Sanger sequenced to identify the incorporation of the ssODN containing the mutation of interest.

**Whole mount in situ hybridization.** 1 dpf larvae were treated with 0.003% phe-nylthiourea to inhibit pigmentation until fixation in 4% paraformaldehyde at 4 dpf. After fixation, larvae were pumiced in microcentrifuge tubes were dehydrated in methanol (5 by 5 min washes while notating) and then stored at -20 °C. To begin in situ hybridization, larvae were rehydrated with 50, 70, and 100% 0.2% PBS-Tween: methanol (5 min washes on a nutator). PBS-Tween washes were then continued (4 min washes while notating). After Proteinase-K treatment, two quick PBS-Tween washes were performed, and then larvae were post-fixed in 4% paraformaldehyde for 20 min while notating. Larvae were then washed with PBS-Tween (5 by 5 min washes on a nutator) and then incubated in hybridization buffer (Hyb-) at 65 °C for at least 1 h in a dry heat block. The riboprobe of interest in Hyb(-) was then incubated with the larvae overnight at 65 °C in a dry heat block. The following day, larvae were washed in 100% Hyb-, 75% Hyb+: 25% 2X SSC-Tween, 50% Hyb+: 50% 2X SSC-Tween, 25% Hyb+: 75% 2X SSC-Tween, all preheated to 65 °C, in a dry heat block. Larvae were then washed with 2X SSC-tween (two washes, 30 min each) and 0.2X SSC-Tween (two washes, 30 min each) at 65 °C in a dry heat block. Larvae were then washed with MAB-Triton-X-100 for 10 min at room temperature while notating and then blocked in blocking solution (2% blocking reagent in MAB + 0.2% Triton-X-100 + 10% sheep serum) for at least 1 h at room temperature on a nutator. Larvae were then treated with anti-Dig AP Fab fragments (1:2000 – Roche 1193927-0901) in blocking solution overnight at 4 °C on a nutator. The following day, larvae were washed with MAB-Triton-X-100 (6 by 30 min washes on a nutator) at room temperature. After a 10 min wash in alkaline phosphatase/NBT (AP)
buffer, larvae were moved to a 24-well plate, covered in aluminum foil to prevent light exposure, and incubated with NBT (2.2 µL mL⁻¹) + BCIP (1.6 µL mL⁻¹) in AP buffer until the reaction completed. After development of the probe, larvae were washed with three quick PBS-Tween washes and then passed through 30, 50, and 70% glycerol washes. A complete protocol with detailed notes is available. The previously characterized riboprobes utilized in this study were msp (GenBank: AV869977.1), and gpr126, originally synthesized with 5'-gaatctgagctgccgtag-3' and 5'-atgctgtgctattcaactcagc-3' primers. For msp, larvae were scored for either presence or absence of signal expression along the PLLn.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The accession number for the coordinates and diffraction data for the Gpr126 (−ns) ECR crystal structure reported in this paper is PDB: 6Y55. The SASBDB IDs for the SAXS experimental data are: SASDFP9, SASDFE9, SDSDFV9, SASDFW9, SASDFX9. The source data underlying Fig. 4b, c, e and Supplementary Figures 1B, 4C, H, and 6b, C are provided as a Source Data file. All other data are available from the corresponding author on reasonable request.

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Author contributions

K.L. cloned, expressed, purified proteins (with assistance from E.F.), carried out bioinformatic and biochemical characterizations, performed crystallography experiments (with assistance from J.L.) and structure determination, performed cAMP-based signaling assays, and collected and analyzed negative-stain EM data (with assistance from M.Z.). R.L.C. and K.R.M. designed and analyzed and R.L.C. performed zebrafish experiments, J.A.R., T.R.S., and K.L. designed and performed SAXS experiments. K.L. performed zebrafish experiments. J.A.R., T.R.S., and K.L. designed and performed SAXS experiments. K.L. and D.A. designed all experiments, interpreted results, and wrote the manuscript.

Competing interests

The authors declare no competing interests.
**Additional information**

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