The Orphan Adhesion-GPCR GPR126 Is Required for Embryonic Development in the Mouse

Helen Waller-Evans¹ᵃ, Simone Prömel¹ᵇ, Tobias Langenhan¹ᶜ, John Dixon², Dirk Zahn², William H. Colledge³, Joanne Doran², Mark B. L. Carlton², Ben Davies⁴, Samuel A. J. R. Aparicio⁵, Johannes Grosse², Andreas P. Russ¹

1 Department of Biochemistry and Magdalen College, University of Oxford, Oxford, United Kingdom, 2 Takeda Cambridge Ltd, Cambridge, United Kingdom, 3 Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom, 4 Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom, 5 Department of Pathology and Laboratory Medicine and BC Cancer Research Centre, University of British Columbia, Vancouver, Canada

Abstract

Adhesion-GPCRs provide essential cell-cell and cell-matrix interactions in development, and have been implicated in inherited human diseases like Usher Syndrome and bilateral frontoparietal polymicrogyria. They are the second largest subfamily of seven-transmembrane spanning proteins in vertebrates, but the function of most of these receptors is still not understood. The orphan Adhesion-GPCR GPR126 has recently been shown to play an essential role in the myelination of peripheral nerves in zebrafish. In parallel, whole-genome association studies have implicated variation at the GPR126 locus as a determinant of body height in the human population. The physiological function of GPR126 in mammals is still unknown. We describe a targeted mutation of GPR126 in the mouse, and show that GPR126 is required for embryonic viability and cardiovascular development.

Introduction

Adhesion-GPCRs are the second largest subfamily of putatively G-protein coupled receptors (GPCR) with more than 30 members in mammals [1]. Their typical domain architecture consists of a C-terminal seven-transmembrane domain homologous to secretin-like GPCRs, and a long N-terminal domain containing a range of protein domains found in cell adhesion proteins [2]. N- and C-terminal domains can be autocatalytically cleaved at the membrane-proximal GPS (GPCR proteolytic site) domain, which is a characteristic feature of this receptor class [3].

Although there is no consensus yet about the physiological function of Adhesion-GPCRs and their molecular mechanism of signalling, the existing data suggest that this receptor class mediates essential cell-cell and cell-matrix interactions [2].

Recently, an orphan receptor of the Adhesion-GPCR family, GPR126, has been shown to play an essential role in the myelination of peripheral nerves by neural crest (NC) -derived Schwann cells in the zebrafish Danio rerio [10]. Schwann cells lacking GPR126 expression fail to to a myelinate their target axons, suggesting that GPR126 is a key component of the elusive signalling pathway by which axons communicate with the myelinating cell [11,12].

In mammals, GPR126 has been described as an orphan receptor with a tightly regulated expression pattern in mouse development (DREG) [13], and has also been isolated from human umbilical vein endothelial cell (HUVEC) cultures (vascular inducible GPCR, VIGR) [14]. Surprisingly, whole-genome association studies have identified genetic variation at the GPR126 locus as a determinant of trunk length and body height in the human population [15,16,17]. The physiological function of GPR126 in mammals is unknown, and there is currently no model that could explain the molecular mechanism of this trait.
We describe here a targeted mutation of GPR126 in the mouse and show that the receptor is required for embryonic viability and cardiovascular development.

**Results**

**GPR126 is an evolutionary innovation specific for vertebrates**

The GPR126 locus is highly conserved in vertebrates. We could identify putative genes encoding homologous proteins with the typical domain architecture (CUB, Lammin G/Pentaxin, GPS, 7TM2, Fig. 1a) in all vertebrate species that were investigated. The synteny of the genomic region surrounding these genes is conserved in fish, birds, reptiles, and mammals, indicating that the predicted genes are true orthologues (Fig. 1b). A GPR126 homologue is also present in the amphibian *Xenopus tropicalis*, but although synteny also appears to be conserved a comprehensive annotation is currently not possible due to the fragmentation of sequence contigs.

The genomes of the sea urchin *S. purpuratus* and of *Trichoplax adhaerens* are predicted to encode Adhesion-GPCRs with N-termini containing a CUB domain in conjunction with EGF or Ig-like domains, respectively. Thus, the domain architectures of these putative receptors are clearly divergent from vertebrate GPR126. No orthologues of GPR126 were found in the high quality genomes of the chordates *Ciona intestinalis*, *C. savignyi* and *Branchiostoma floridae*, or in the draft genome assembly of the lamprey *Petromyzon marinus*.

These results indicate that GPR126 is specific for vertebrates. This would be consistent with the proposed function in Schwann cell precursors (SCP) [10] which originate from the NC. The NC is considered to be a developmental innovation that is unique to vertebrates [18].

The closest paralog of GPR126 is GPR112, which also appears to be specific for vertebrates (Fig. 1a). In mammals, GPR112 is located on the X chromosome and contains a long (>1500 amino acids) domain in its N-terminus predicted to be an extended coil structure. This domain is not present in GPR126. In zebrafish, GPR112 is located on Chromosome 10, is lacking the coil domain, and is highly similar to zebrafish GPR126 (Chr. 20, amino acid identity 30%, similarity 48%, Figure S1).

**A targeted mutation of GPR126 expressing a LacZ reporter gene**

To follow the expression pattern of the wild-type GPR126 in detail and to identify its physiological function we generated a targeted mutation in murine embryonic stem cells which disrupts the GPR126 coding region and expresses a LacZ reporter gene under the control of the GPR126 promotor (GPR126LacZ). The sequences encoding part of the 7TM domain were deleted (Fig. 1a), and a LacZ reporter gene cassette [19] was inserted into the locus (Fig. 2a, b).

Heterozygous GPR126LacZ/+ carriers were born at the expected Mendelian frequency (Table 1) and did not show any obvious phenotypes, indicating that the targeted locus does not have detrimental dominant activity. Wild-type GPR126 transcript could not be detected in homozygous embryos (see below), suggesting that the targeted allele is a null mutation (Fig. 2c).

**Dynamic segmental expression of GPR126 in the embryo**

We investigated the expression pattern of the wild-type GPR126 gene in adult tissues by RT-PCR, *in situ* hybridization (ISH), and immunofluorescence staining (IF). Our PCR analysis confirmed that GPR126 mRNA is widely expressed (Figure S2) [13]. However, in spite of substantial efforts we were not able to reproducibly detect the wild-type gene product on a cellular level by ISH or IF.

In heterozygous GPR126LacZ/+ embryos, no reporter gene expression was seen up to 9.5 dpf (days post fertilisation). At 10.5 dpf a weak segmental expression is consistently observed in trunk and tail (Fig. 3 a, g) [13]. This pattern becomes more pronounced at 11.5 dpf, and rapidly fades at 12.5 dpf (Fig. 3 c, h–j). Sectioning of embryos shows the expression in close proximity to the somitic myotome, but very little LacZ expression overlaps with cells positive for a myotome marker α-smooth muscle actin (α-SMA) (Fig. 4 a, b, d, e, g, h). Notably, expression of GPR126 in each segment is restricted to very few cells in a dispersed "salt-and-pepper" fashion (Fig. 3 g, 4b). The time course of expression observed in our study is slightly delayed compared to the ISH data reported earlier [13]. This most likely reflects the time required for expression and maturation of the reporter protein.

The pattern in the embryo is consistent with a very dynamic expression in a small subset of neural crest derived cells in trunk and tail. The typical locations of the cranial neural crest and the branchial arches including cardiac neural crest are negative for reporter expression. We also detected expression of GPR126LacZ in the trophoblast giant cells (TGC) of the placenta (Fig. 5).

We were not able to detect clear and reproducible expression in the CNS, branchial arches and cephalic mesenchyme, the heart, vasculature, or other organ primordia of GPR126LacZ/+ embryos. Analysis of specimens from adult heterozygous transgenics also did not reveal distinct expression patterns of GPR126 in mature tissues. We were not able to detect a staining pattern equivalent to the expression in SCP in Zebrafish [10].

**Loss of GPR126 function leads to mid-gestation lethality**

No homozygous offspring are born from matings of heterozygous carriers, indicating embryonic lethality (Table 1, 2). The genotypes of dissected embryos show that a sharp loss of viability occurs from 10.5 dpf to 12.5 dpf, consistent with the onset of intra-embryonic expression. At 11.5 dpf more than 50% (23/43) and at 12.5 dpf 80% (12/15) of the homozygous embryos are dead. No viable embryos were recovered at 13.5 dpf (Table 2).

Mutant embryos show no obvious malformations and somite numbers are indistinguishable from controls, indicating that development is not significantly delayed at 10 dpf and 11 dpf (not shown). We did not detect abnormalities in the histological architecture of the placenta of homozygous embryos or changes in the distribution of TGC (Fig. 5). Thus, we could not find evidence for defects in placental development or function.

The number and distribution of cells expressing the GPR126LacZ reporter gene is changed in homozygous embryos. An increased number of LacZ-positive cells are clustered in a narrow extension of the normal expression domain (Fig. 3 d–f, l). Histological sections confirm the presence of a larger number of LacZ-positive cells in the somitic domain, and again indicate that only a minority of these co-express α-SMA (Fig. 4 c, f, i). This observation would be consistent with a complete or partial block in the differentiation and/or migration of a GPR126-expressing cell population, leading to an accumulation of cells that might be delayed or stalled on their path of migration.

**Cardiovascular failure in GPR126 mutants**

Viable homozygotes with beating hearts are regularly found in the same litter next to dead embryos showing signs of circulatory failure like congestion, edema, and internal hemorrhage. This suggests that cardiovascular function in mutants is initiated normally and fails within 24–48 hrs after the onset of GPR126 expression.
Figure 1. The domain architecture and genomic conservation of GPR126. a. The domain architecture of GPR126 in comparison to other Adhesion-GPCRs. Sketches are drawn to scale of primary protein structure, the conserved domains of the N-terminus are colour-coded. The shaded box in mouse GPR126 depicts the region deleted in the targeted allele GPR126\textsuperscript{lacZ}. The arrow in zebrafish GPR126 shows the point of truncation in \textit{gpr126(st49)} [10]. The closest homolog of GPR126 is GPR112, which shares the Pentaxin domain but lacks the CUB domain. In zebrafish, GPR126 and GPR112 are structurally more similar than in mammals, where GPR112 contains a very long coiled domain in its N-terminus. CUB: C1r/C1s, Uegf, Bmp1 domain; EGF: Epidermal Growth Factor domain; HRM: Hormone-binding motif. b. Schematic depiction of the conserved synteny surrounding the GPR126 locus in diverse vertebrate species. Note that the synteny of the zebrafish locus is highly conserved to reptiles, birds, and mammals, while other fish species show higher divergence. NMBR: neuromedin B receptor; GJE-1: gap junction protein epsilon 1 (pseudogene \textit{gje-1} in humans); VTA-1: Vps20-associated 1 homolog (\textit{S. cerevisiae}); HIVEP-2: HIV enhancer binding protein 2; AIG-1: androgen-induced gene 1; E2F6: E2F transcription factor 6; MSRA: methionine sulfoxide reductase A; ASAP2; ArfGAP with SH3 domain, ankyrin repeat and PH domain 2; ITGB1BP1: integrin beta 1 binding protein 1; EDN-1: endothelin 1; NACHT: NACHT-NTPase containing protein; KIAA1737: unknown novel protein; ZNF410: zinc-finger protein 410. doi:10.1371/journal.pone.0014047.g001
In wild-type embryos, crucial steps of cardiac development like septation and the formation of the outflow tract are only completed after 12.5 dpf. As embryos homozygous for the GPR126\textsuperscript{LacZ} allele die before this time point it is difficult to distinguish primary defects in heart development from secondary effects of circulatory failure due to other reasons. Expression of GPR126 could not be detected in typical cardiac neural crest (CNC) locations \cite{20}, and histological analysis indicates that septation and the formation of the CNC-derived outflow tract are initiated normally (Fig. 6).

The development of the ventricular wall is not obviously defective at 10.5 dpf, and myocardial trabeculation is formed. In homozygous mutant embryos that still showed active circulation at the time of dissection we could not detect an obvious reduction of myocardial tissue or a lack of trabeculation. However, in embryos that were dissected after onset of circulatory failure we found a pronounced thinning of the myocardial wall (Fig. 6c, d). This suggests that ventricular function is initially normal and might fail under increased load.

In heterozygous carriers of the GPR126\textsuperscript{LacZ} allele reporter gene expression in the ventricular wall can hardly be detected above background levels. However, the examination of myocardial tissue from homozygous mutants revealed a dispersed population of cells with a very low but reproducible level of LacZ expression (Fig. 6e). This suggests the presence of a GPR126-expressing cell population in myocardial tissue, and provides a possible rationale for cardiac failure in mutants. Counterstaining established that as in the somite only a subset of LacZ positive cells also show \alpha-SMA expression, while no overlap could be detected with the endothelial marker PECAM (Fig. 6 f–h).

The difference in myocardial lacZ staining between heterozygotes and homozygous mice could be due to the increased gene dosage in homozygotes, or due to an accumulation of lacZ positive cells in mutants. Counterstaining established that as in the somite only a subset of LacZ positive cells also show \alpha-SMA expression, while no overlap could be detected with the endothelial marker PECAM (Fig. 6 f–h).

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Table 1. Live offspring from GPR126\textsuperscript{LacZ} matings.

| Stage | Cross | \(+/-\) | \(+/-\) | \(-/-\) | \ N \ |
|-------|-------|--------|--------|--------|--------|
| weanlings | +/- x wt | 186 | 49.3% | 191 | 50.7% | n/a | 377 |
| weanlings | +/- x +/- | 10 | 32.3% | 21 | 67.7% | 0 | 0.0% | 31 |

Intercrosses of animals heterozygous for GPR126\textsuperscript{LacZ} do not produce liveborn homozygous offspring.

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Vascular development in GPR126 mutants

GPR126 was initially isolated from HUVEC cultures that had been challenged with lipopolysaccharides or thrombin \cite{14} and
was implicated in endothelial cell function. We observed intra-
embryonic hemorrhage in ~50% (24/49) of GPR126
LacZ embryos that were found dead at dissection, and therefore
investigated defects in vascular development as a possible cause
of lethality. Immuno-histochemical detection of the endothelial
marker PECAM-1 in whole-mount embryos and in histological
sections did not reveal any consistent defects of angiogenesis or
vasculogenesis in mutant embryos (Fig. 7). We could not detect co-
expression of GPR126LacZ and PECAM, indicating that the
expression level is below the detection limit in unchallenged
endothelial cells.

The development of endothelium in large and small vessels
(Fig. 7 a, b, e, f) and the complexity of the vascular tree (Fig. 7 c, d)
are not significantly different between homozygous mutants and
controls. No abnormalities were detected in placental vessels and
umbilical cord. The paired dorsal aortae develop and fuse
normally (Fig. 7 g, h), and the vascular wall is stabilised by the
recruitment of smooth muscle cells (Fig. 7 i, j) and extracellular
matrix. Hematopoietic clusters in mutant vessels also develop at
the correct time and localisation (not shown). Thus, in spite of the
reported expression of GPR126 in endothelial cell cultures we
could not detect expression of GPR126 in endothelia in vivo, and
were not able to find evidence supporting a defect of vascular
development as the cause of embryonic lethality.

**Discussion**

We describe the first genetic analysis of GPR126 function in a
mammalian model. The targeted mutation GPR126LacZ deletes
part of the 7TM region (Fig. 1a) and is presumably a null allele.
Residual wild-type transcripts could not be detected in mutant
embryos, and a truncation of the related receptor lat-1 has been
shown to lack residual function in C. elegans [9,23].

Homozgous mutant embryos show fully penetrant lethality due to
cardiovascular failure at mid-gestation. Lethality coincides with the
expression of GPR126LacZ in a small group of cells in trunk and tail
somites, but due to the very low and transient levels of gene expression
we could not directly determine which anatomical structures are
ultimately derived from this cell population. We did not find evidence
for developmental defects of the vascular system, the cardiac neural
crest, or the placenta, and suggest that GPR126 is required for the
development and/or the function of nascent myocardial tissue.

Figure 3. The expression pattern of the GPR126LacZ reporter allele. Expression pattern of GPR126. Embryos carrying the GPR126LacZ allele
were stained for β-galactosidase activity as whole-mount preparations. Heterozygous (a–c) and homozygous (d–f) embryos at 10.5, 11.5, and 12.5 dpf,
respectively. (g–j): higher magnification view of expression in somitic region in a heterozygous embryo. Note the faint and transient expression. (k, l):
comparison of heterozygous and homozygous embryo at 11.5 dpf. Note the change in expression pattern and intensity in the homozygous
specimen. Scalebar (a–f) 2 mm, (g–l) 500 µm.
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Expression of human GPR126 has been reported in vitro in HUVEC cultures stimulated by lipopolysaccharide (LPS) [14]. However, we could not detect GPR126\(^{\text{LacZ}}\) in embryonic endothelia in vivo and did not observe morphological abnormalities of the developing vascular system. A defect of the cardiac neural crest in GPR126 mutants also appears unlikely. Firstly, CNC cells migrate via the branchial arches [24], where GPR126 is not expressed. Secondly, the development of the cardiac outflow tract is initiated normally in GPR126 homozygotes. Thirdly, mice with severe disruption of CNC development, e.g. Splotch mutants, show malformation of the outflow tract by 13.5 dpf and embryonic death by 14.5 dpf [24], significantly later than GPR126 mutants. This strongly suggests that abnormal development of the CNC would not be sufficient to explain the phenotype of GPR126\(^{\text{LacZ}}\) homozygotes.

Although GPR126 is expressed in TGC, the histological architecture of the placenta is normal in GPR126 homozygotes. Embryonic development up to 11 dpf is not delayed, arguing against a significant impairment of placental function. We could not find precedents in the literature where a functional defect in a morphologically normal placenta causes fully penetrant lethality in a narrow time window at 11.5–12.5 dpf, as observed in GPR126 mutants. The lethality profile is the same on inbred and outbred genetic backgrounds, indicating that a loss of immune privilege is not a likely cause of embryonic death. In future experiments, the role of GPR126 in the placenta could be directly tested by creating chimeras of homozygous GPR126 embryos with tetraploid wild-type embryos.

Weakly LacZ-positive cells were observed in the ventricular wall of GPR126\(^{\text{LacZ}}\) homozygotes, suggesting that GPR126 might have a function in myocardial development. In GPR126 mutants the myocardial wall is thinned, but shows no defect in trabeculation. Thus, a defect in the development of the ventricle wall of the heart appears to be the most likely cause of death in GPR126 mutants.

In contrast to the embryonic lethality in GPR126\(^{\text{LacZ}}\) mice, zebrafish homozygous for the gpr126(st49) allele develop into fertile adults with a defect in Schwann cell function [10]. The predicted products of GPR126\(^{\text{LacZ}}\) and gpr126(st49) are similar (Fig. 1a) [10], arguing against an allele-specific effect. Due to the early embryonic lethality we could not investigate the role of GPR126 in mouse SCP and myelin sheath formation. However, it is unlikely that a defect in SCP causes lethality in GPR126\(^{\text{LacZ}}\) mice, as other mutants lacking SCP develop to term [25].

Our results indicate that GPR126 has an essential function in mammalian development that has not been predicted by the analysis of GPR126 mutations in Zebrafish. This could either be due to true differences in the developmental pathways of fish and mammals, or due to different levels of functional redundancy and compensation by other members of the Adhesion-GPCR family.

A link between heart morphogenesis and Schwann cell development is demonstrated in mice mutant for components of the neuregulin/erbB2-4 pathway, which display a syndrome of myocardial failure and defects in SCPs [26,27,28]. In zebrafish, the mutant phenotypes are milder with respect to embryonic lethality, presumably due to the existence of additional paralogs, e.g. two copies of erbB3 (erbB3a and erbB3b [29,30]). This suggests that the underlying developmental pathways are similar between fish and mammals, and that partial genomic redundancy is the cause of phenotypic differences.

The conserved synteny at the GPR126 locus clearly indicates that mouse and zebrafish GPR126 are true orthologs. We could not detect an additional copy of GPR126 in the zebrafish genome which could explain the milder phenotype of the gpr126(st49) allele. However, the most closely related Adhesion-GPCR, GPR112, shows much higher homology to GPR126 in fish than in mammals (Fig. 1a) [10]. This suggests that in zebrafish a partial redundancy between fish and mammals, or due to true differences in the developmental pathways of fish and mammals, or due to different levels of functional redundancy and compensation by other members of the Adhesion-GPCR family.

Conditional inactivation of the erbB2 pathway in adult mice leads to dilated cardiomyopathy and has been linked to the cardio toxicity of the therapeutic antibody trastuzumab (Herceptin®) [31]. Our results strongly suggest that any therapeutic intervention targeting GPR126, e.g. to modulate myelination, will require careful consideration of cardiac side effects.

Due to the very restricted and transient expression of GPR126, a limitation of our study has been the difficulty to characterise the origin and fate of GPR126-positive cells. We consider it unlikely that standard ISH and IF will be able to fully resolve this problem. The further analysis of GPR126 function will require the identification of the tissues formed by GPR126-positive cells by permanent transgenic marking, e.g. by constitutive activation of a LacZ reporter gene by a GPR126-Cre transgene [21,22], the use of tissue specific conditional mutagenesis, and the analysis of chimeric embryos.
Figure 5. GPR126 is expressed in the placenta. Gpr126 is expressed in trophoblast giant cells, but the anatomy of the placenta is normal in GPR126$^{LacZ}$ homozygotes. 2 mm vibratome sections of placentas from wild-type, heterozygous and GPR126 null embryos at e10 and e11 were stained with X-Gal to visualise GPR126$^{LacZ}$ expression, cryosectioned at 20 μm and counterstained with Nuclear Fast Red. TGC - trophoblast giant cells, Sp - spongiotrophoblast, Lab - labyrinth. Scalebar = 100 μm. doi:10.1371/journal.pone.0014047.g005

Table 2. Embryonic genotypes derived from GPR126$^{LacZ}$ matings.

| Stage | Cross   | +/+ | +/- | -/- | resorption | N  |
|-------|---------|-----|-----|-----|------------|----|
| dpf   | genotypes | n(dead) | %  | n(dead) | %  | n(dead) | %  | n | %  |
| 9.5   | +/- x +/- | 15 | 23.4% | 33 | 51.6% | 14 | 21.9% | 2 | 3.1% | 64 |
| 10.5  | +/- x +/- | 50 | 24.5% | 104 | 51.0% | 35 | 17.2% | 15 | 7.4% | 204 |
| 11.5  | +/- x +/- | 51(3) | 23.6% | 95(4) | 44.0% | 43(3) | 19.9% | 27 | 12.5% | 87 |
| 12.5  | +/- x +/- | 25(0) | 22.1% | 64(1) | 56.6% | 15(12) | 13.3% | 9 | 8.0% | 87 |
| 13.5  | +/- x +/- | 19(0) | 21.8% | 48(2) | 55.2% | 2(2) | 2.3% | 18 | 20.7% | 87 |

Genotyping of embryos dissected after timed matings reveals a sharp loss of homozygous offspring from 10.5 dpf onwards. doi:10.1371/journal.pone.0014047.t002
Genetic variation at the GPR126 locus is linked to the heritability of height in the human population [15,16,17]. The molecular nature of different GPR126 alleles is not known. GPR126 contributes specifically to trunk length rather than limb length, consistent with our finding that the gene is expressed in a segmental pattern in a putative migratory cell population. While our findings cannot explain the role of GPR126 polymorphisms in human height, our finding that the gene is expressed in a putative migratory cell population suggests that GPR126 may play a role in the development of the trunk and possibly the limb.
GPR126 Knockout Mouse

Figure 7. The developing vasculature in GPR126ΔlacZ embryos. Patterning of blood vessels is normal in GPR126ΔlacZ embryos. Wild-type (a, c, e, g, i) and homozygous GPR126ΔlacZ (b, d, f, h, j) embryos at 10.5 dpf were stained as whole-mount specimens with an α-PECAM (a–h) or α-SMA (i, j) antibodies. (a, b) Overall patterning of the vasculature is normal in GPR126ΔlacZ embryos. (c, d) The complexity of branching of cranial vessels is normal, as is the development of intersomitic vessels (e, f). (g, h) The dorsal aortae form normally and smooth muscle cells are recruited to stabilize the vessels (i, j). Scalebar1 mm (a, b) 200 μm (e, f) and 50 μm (g–j).

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GGATCTTCCAAGGAAGTGCCCTCAG AA-3′ for mGPR126, and 5′- GCAGCGCATCGCCTTCTATC-3′ for the neomycin resistance cassette.

Whole-mount embryo staining

Histological staining for LacZ reporter gene activity was performed as described [19]. Adult tissues or embryos dissected after timed matings were washed twice for 5 min in PBS containing 2 mM magnesium chloride, fixed at 4°C in 0.2% (v/v) glutaraldehyde, 100 mM phosphate buffer pH 7.4, 5 mM EGTA, 2 mM magnesium chloride, and washed three times for 5 min at room temperature in LacZ Wash Buffer (100 mM phosphate buffer pH 7.4, 0.01% (v/v) Nonidet P-40, 0.02% (w/v) sodium deoxycholate, 2 mM magnesium chloride). Embryos and tissues were incubated for 15–48 h in LacZ stain (0.8–1.6 mg/ml X-Gal, 100 mM phosphate buffer pH 7.4, 0.01% (v/v) Nonidet P-40, 0.02% (w/v) sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride) at 37°C in the dark. Samples were post-fixed in 4% (w/v) paraformaldehyde in PBS and Histology.

For whole-mount immunohistochemistry, embryos were stained with primary antibodies against PECAM and α-SMA (rat anti-mouse PECAM clone MEC13 (BD Pharmingen) at a 1 in 200 dilution and mouse anti-human α-SMA clone 1A4 (Dako) at a 1 in 500 dilution) and HRP conjugated secondary antibodies against rat and mouse (goat anti-rat IgG (Stratech) at a 1 in 100 dilution and goat anti-mouse IgG (Millipore) at a 1 in 100 dilution). Embryos were fixed in 4% (w/v) paraformaldehyde in PBS, dehydrated through a series (25%, 50%, 75%, 100%, 100% (v/v)) of methanol in PBS, and stored at −20°C.

Specimens were bleached in 6% (v/v) hydrogen peroxide in methanol for 5 h 30 min on a shaking platform, rehydrated through a series (100%, 75%, 50%, 25% (v/v)) of methanol in PBS, permeabilised in PBS containing 0.1% (v/v) Triton X-100, and blocked in blocking solution (PBS containing 2% (w/v) Marvel skimmed milk powder and 0.1% (v/v) Triton X-100) for 1 h before incubation in rat anti-mouse PECAM clone MEC13 primary antibody (BD Pharmingen) at a 1 in 200 dilution in blocking solution. Specimens were then incubated twice in blocking solution for 1 h at 4°C and three times for 1 h at room temperature before incubation in HRP conjugated goat anti-rat IgG secondary antibody (Stratech) at a 1 in 100 dilution in blocking solution overnight at 4°C. After further incubations in blocking solution for 1 h at 4°C and three times for 1 h at room temperature before further blocking in PBS containing 0.2% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100, once for 10 min at room temperature, once for 40 min at room temperature, the stain was developed with PBS containing 0.5 mg/ml DAB, 2 mg/ml ammonium nickel sulphate and 0.003% (v/v) hydrogen peroxide for 2–10 min.

Histology

For frozen sections, samples were cryoprotected in 30% (w/v) sucrose in PBS and embedded in Tissue Tek (Bayer). 10–20 μm sections were taken using a Bright 5040 cryostat. For paraffin-embedded sections, 6 μm sections were taken on a Leica DSC1 microtome and mounted onto X-tra adhesive slides (Surgipath). Haematoxylin/eosin staining was performed according to standard protocols.

Immunohistochemistry was performed according to standard protocols with the primary antibodies α-GPR126 (Abcam) 1:50, 1:100 and 1:500 dilution, α-PECAM (platelet and endothelial cell adhesion molecule) clone MEC13 (BD Pharmingen) 1:200 dilution, α-smooth muscle actin (α-SMA) clone 1A4 (Dako) 1:500 dilution. Signal was detected with secondary antibodies conjugated to horseradish peroxidase (goat anti-rabbit IgG (Millipore), goat anti-mouse IgG (Vector Laboratories), or a fluorophore (Alexa Fluor 488 conjugated goat anti-mouse IgG (Invitrogen), all in 1:100 dilution. Sections were counterstained with Nuclear Fast Red or Haematoxylin and mounted using Vectamount or Vectashield (Vector Laboratories).

Microscopy

Histological sections were visualised with a Zeiss Axioplan2.0 microscope fitted with an AxioCam HD camera. Whole-mount samples were documented with a Nikon SMZ1500 dissection microscope fitted with a Leica DC500 camera. Images were collected and manipulated using Adobe Photoshop 9.0.
Supporting Information

Figure S1 Alignment of zebrafish GPR126 and GPR112, showing the high degree of homology between the paralogs. Found at: doi:10.1371/journal.pone.0014047.s001 (0.66 MB TIF)

Figure S2 Expression of Gpr126 in adult tissues. cDNA was amplified from wild-type mouse organs using gene specific primers against Gpr126. β-Actin was amplified as positive control to normalize cDNA concentration (β-Actin + RT (reverse transcriptase); β-Actin -RT: reaction without reverse transcriptase as control for genomic DNA contamination. Expected band sizes: Gpr126 759bp, β-Actin 303bp.

References
1. Bjarnadóttir T, Fredriksson R, Schiöth H (2007) The Adhesion GPCRs: A unique family of G protein-coupled receptors with important roles in both central and peripheral tissues. Cell Mol Life Sci.
2. Yona S, Lin H-H, Sui WO, Gordon S, Stacey M (2008) Adhesion GPCRs: emerging roles for novel receptors. Trends Biochem Sci 33: 491–500.
3. Lin H-H, Chang G-W, Davies JQ, Stacey M, Harris J, et al. (2004) Autocatalytic cleavage of the EMR2 receptor occurs at a conserved G protein-coupled receptor proteolytic site motif. J Biol Chem 279: 31823–31832.
4. Weston MD, Luijendijk MWJ, Humphrey KD, Moller C, Kimberling VJ (2004) Mutations in the VGLR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II. Am J Hum Genet 74: 357–366.
5. Piao X, Hill RS, Bodeli A, Chang BS, Basel-Vanagaite L, et al. (2004) G protein-coupled receptor-dependent development of human frontal cortex. Science 303: 2033–2036.
6. Lawrence PA, Smulh G, Casal J (2007) Planar cell polarity: one or two pathways? Nat Rev Genet 8: 555–563.
7. Strutt D (2008) The planar polarity pathway. Curr Biol 18: R398–902.
8. Tsirir F, Bar I, Jossin Y, De Backer O, Goffinet A (2005) Protocadherin Celsr3 is crucial in axonal tract development. Nat Neurosci 8: 451–457.
9. Langenhani T, Promel S, Mestek L, Emaarci B, Waller-Evans H, et al. (2009) Latrophilin signaling links anterior-posterior tissue polarity and oriented cell divisions in the C. elegans embryo. Dev Cell 17: 494–504.
10. Monk KR, Naylor SG, Glenn TD, Mercurio S, Perlin JR, et al. (2009) A G protein-coupled receptor is essential for Schwann cells to initiate myelination. Science 325: 1402–1405.
11. Aguayo AJ, Kasarjian J, Skamene E, Kongshavn P, Bray GM (1977) Myelination of mouse axons by Schwann cells transplanted from normal and abnormally human nerves. Nature 268: 753–755.
12. Sherman DL, Brophy PJ (2005) Mechanisms of axon ensheathment and myelin growth. Nat Rev Neurosci 6: 603–609.
13. Morishita T, Haraguchi K, Ueda N, Okada M, Furuya T, et al. (2004) DREG, a developmentally regulated G protein-coupled receptor containing two conserved proteolytic cleavage sites. Genes Cells 9: 549–560.
14. Stohlík C, Krivomáry R, Dolflатель A, Binder B, Lippr J (2004) VGR--a novel inducible adhesion family G-protein coupled receptor in endothelial cells. FEBS Lett 569: 149–155.
15. Hancock DB, Engjeshaj M, Will J, Gharbi SA, Loeber LR, et al. (2009) Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. Nat Genet.
16. Zhao J, Li M, Bradfield JP, Zhang H, Mimch FD, et al. (2010) The role of height-associated loci identified in genome wide association studies in the determination of pediatric stature. BMC medical genetics 11: 96.
17. Soranzo N, Rivadeneira F, Chiappeen-Horley U, Malkina I, Richards JB, et al. (2009) Meta-analysis of genome-wide scans for human adult stature identifies novel Loci and associations with measures of skeletal frame size. PLoS Genet 5: e1000445.
18. Shinfeld SM, Holland PW (2000) Vertebrate innovations. Proc Natl Acad Sci USA 97: 4449–4452.
19. Rusu AP, Wattler S, Collègde WH, Aparicio SA, Carlton MB, et al. (2000) Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature 404: 95–99.
20. Creazzo TL, Godt RE, Leatherbury L, Conway SJ, Kirby MI (1998) Role of cardiac neural crest cells in cardiovascular development. Ann Rev Physiol 60: 267–286.
21. Chai Y, Jiang X, Ito Y, Bringas P, Han J, et al. (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127: 1671–1679.
22. Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM (2000) Fate of the mammalian cardiac neural crest. Development 127: 1607–1616.
23. Vakonakis I, Langenhani T, Promel S, Russ A, Campbell ID (2008) Solution structure and sugar-binding mechanism of mouse latrophilin-1 RBL, a 7TM receptor-attached lectin-like domain. Structure 16: 944–953.
24. Conway SJ, Henderson DJ, Cop C (1997) Fas3 is required for cardiac neural crest migration in the mouse: evidence from the spleoth (Sp2) mutant. Development 124: 505–514.
25. Woldeyesus MT, Britch S, Riedmacher D, Xu L, Sonneneberg-Riedmacher E, et al. (1999) Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. Genes Dev 13: 2538–2548.
26. Gassmann M, Casagrande F, Orioli D, Simon H, Lai C, et al. (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 378: 394–398.
27. Lee KP, Simon H, Chen H, Bates B, Hung MC, et al. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature 378: 394–398.
28. Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. Nature 378: 396–398.
29. Budi EH, Patterson LB, Parichy DM (2008) Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation. Development 135: 2603–2614.
30. Lainey JAGC, Braasch I, Walter RB, Meierjohann S, Scharf M (2010) Lineage-specific co-evolution of the Egf receptor/ligand signaling system. BMC Evol Biol 10: 27.
31. Ozelik C, Erdmann B, Pla B, Wetscherek N, Britsch S, et al. (2002) Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. Proc Natl Acad Sci USA 99: 8880–8885.
32. Cash JL, Hart R, Rux A, Dixon JPC, Collègde WH, et al. (2008) Synthetic chemerin-derived peptides suppress inflammation through ChemR23. J Exp Med 205: 767–775.