Adrenergic activation modulates the signal from the Reissner fiber to cerebrospinal fluid-contacting neurons during development

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Abstract
The cerebrospinal fluid (CSF) contains an extracellular thread that is conserved in vertebrates and referred to as the Reissner fiber. Genetic ablation in the zebrafish revealed that the Reissner fiber controls body axis morphogenesis in the embryo. Yet, the signaling cascade originating from this fiber to ensure body axis straightening is not fully understood. Here, we explore the functional link between the Reissner fiber and undifferentiated spinal neurons contacting the CSF (CSF-cNs). First, we show that the Reissner fiber is required for the expression of urp2, a neuropeptide expressed in CSF-cNs. Using in vivo calcium imaging, we show that the Reissner fiber is also required for embryonic calcium transients in these spinal neurons. Finally, we study how local adrenergic activation directly in the CSF can substitute for the Reissner fiber-signaling pathway to CSF-cNs and rescue body axis morphogenesis. Our results suggest that the Reissner fiber acts on CSF-cNs to establish body axis morphogenesis by controlling the availability of a chemical signal in the CSF.
**Introduction**

One of the major questions in the study of multicellular organism development is to understand how precise morphogenesis is ensured during embryonic and postembryonic stages while the animal grows into an adult. In particular, this process requires a coordination between cell specification signals and the control of the tissue shape (Chan et al., 2017). It has recently emerged that the cerebrospinal fluid (CSF) contains many signals important for cell differentiation, and is an important route for the control of morphogenesis (Fame and Lehtinen, 2020). The CSF is a complex liquid filling the central nervous system cavities containing a set of diffusible signaling cues guiding neurogenesis (Lehtinen et al., 2011) and brain morphology in a tissue autonomous manner (Kaiser et al., 2019; Langford et al., 2020). In numerous vertebrate species, the CSF circulation is in part generated by the coordinated movement of cilia projecting to the lumen of brain ventricles and central canal of the spinal cord (Faubel et al., 2016; Hagenlocher et al., 2013; Olstad et al., 2019; Sternberg et al., 2018; Thouvenin et al., 2020).

The role of cilia in the control of CSF composition and circulation has gained a special attention in zebrafish recently. Indeed, disruption of cilia motility in this species has been long known to induce a typical phenotype consisting in a downward curvature of the posterior axis of the embryo (Brand et al., 1996; Jaffe et al., 2016; Kramer-Zucker et al., 2005; Sullivan-Brown et al., 2008). This phenotype has recently been linked to a role of the CSF in body axis curvature establishment and maintenance downstream of cilia function (Cantaut-Belarif et al., 2018; Grimes et al., 2016). We previously showed that a specific component of the CSF is important for axis morphogenesis in the zebrafish embryo: the Reissner fiber (RF) (Cantaut-Belarif et al., 2018). The Reissner fiber (Reissner, 1860) is an acellular proteinous thread bathing in the brain and spinal cord cavities early in development (Troutwine et al., 2019), formed by the aggregation of the SCO-spondin glycoprotein, secreted into the CSF by the subcommissural organ and the floor plate (Lehmann and Naumann, 2005; Meiniel et al., 2008). This fiber fails to assemble in cilia-defective mutants, and scospondin mutant embryos lacking the Reissner fiber exhibit a curled-down axis despite exhibiting normal cilia motility and CSF flow (Cantaut-Belarif et al., 2018; Rose et al., 2020). These observations suggest that the fiber itself plays a role in the control of body axis morphogenesis in the embryo, and that its absence causes a curled-down phenotype, including in cilia-defective mutants. However, the signal linking the presence of the Reissner fiber and its long-range effect on body axis morphogenesis is not fully understood.

Recent studies suggest that this signal relies on the expression of Urp1 and Urp2 peptides and is driven by CSF flow (Lu et al., 2020; Zhang et al., 2018). These peptides
belong to a family of neuropeptides similar to Urotensin-II, related to Somatostatin and conserved throughout vertebrate species in which they are described to have endocrine, cardiac and neurophysiological functions (Vaudry et al., 2015). In the zebrafish embryo, urp1 and urp2 are expressed along the antero-posterior axis of the neural tube in spinal sensory neurons (Quan et al., 2015) called CSF-contacting neurons (CSF-cNs). These conserved interoceptive neurons form two populations of different developmental origins that are located ventrally and dorso-laterally relative to the central canal of the zebrafish spinal cord (Djenoune et al., 2017; Park et al., 2004). CSF-cNs extend into the CSF a microvilliated apical extension that starts differentiating in the embryo and becomes fully mature in the larva to tune the mechanosensory responses of CSF-cNs upon tail bending (Desban et al., 2019). Recently, the Reissner fiber was shown to be required for the mechanosensory function leading to intracellular calcium increase in differentiated CSF-cNs (Orts-Del’Immagine et al., 2020). At the embryonic stage, undifferentiated CSF-cNs that are located ventrally in the neural tube exhibit spontaneous calcium transients at rest, which are abolished in cilia-defective mutants devoid of a proper Reissner fiber (Sternberg et al., 2018).

In this study, we aimed to better understand the signal linking the Reissner fiber to body axis morphogenesis. To investigate whether the Reissner fiber is required for a signal towards the immature CSF-cNs, we sought to decipher the nature of this signal and how it affects at long distance body axis straightening. We show that the Reissner fiber is required for a signal controlling urp2 but not urp1 expression. Using in vivo calcium imaging, we report that the Reissner fiber is also required for calcium signaling in urp2-expressing ventral CSF-cNs, confirming the existence of a crosstalk between the Reissner fiber and undifferentiated CSF-cNs at the embryonic stage. Using the pkd2l1 mutant, we show that the loss of calcium signaling in ventral CSF-cNs does not affect urp2 expression nor body axis curvature. Finally, we show that local injections of monoamines in the brain ventricles of scospondin mutants restore the Reissner fiber-dependent signal together with body axis straightening. Our work demonstrates that the Reissner fiber-dependent signal to ventral CSF-cNs contributes to body axis straightening and is modulated by adrenergic ligands.
Results and Figures

The Reissner fiber controls urp2 but not urp1 gene expression

To explore the signals downstream the Reissner fiber, we performed a transcriptomic analysis of scospondin mutants (Figure 1).

A

onset of body axis defects

control siblings

spinal cord

Reissner fiber

homozygous mutants

scospondin^{icm13/icm13} spinal cord

scospondin^{icm15/icm15} spinal cord

30 hpf

phenotype categorization & whole RNA extraction

RNAseq

parwise comparison

scospondin^{icm13/icm13} vs. control siblings &

scospondin^{icm15/icm15} vs. control siblings

common hits & qRT-PCR validation

48 hpf

B urp1

- control siblings
- scospondin^{icm15/icm15}

C urp2

- control siblings
- scospondin^{icm15/icm15}

normalized expression

30 hpf 48 hpf

ns ns

* **
Figure 1. The Reissner fiber is required for urp2 but not urp1 gene expression.

(A) Schematic of the experimental design. Embryos obtained from scospondin\textsuperscript{icm15/+} or scospondin\textsuperscript{icm13/+} crosses were raised until 48 hpf and categorized according to their external phenotype: straight body axis (control siblings, top) or curled-down body axis (homozygous mutants, bottom) prior to RNA extraction. RNA sequencing was then performed on 3 independent replicates for each allele and allowed pairwise comparisons of transcriptomes to identify commonly regulated genes. qRT-PCR experiments were performed to validate transcriptomic data at the onset of body axis defects (30 hpf) induced by the loss of the Reissner fiber and at the same stage as the RNA sequencing where the phenotype is fully developed (48 hpf). Null scospondin\textsuperscript{icm13/icm13} and hypomorphic scospondin\textsuperscript{icm15/icm15} mutant embryos share the same peculiar curled-down phenotype induced by the loss of the Reissner fiber in the central canal of spinal cord (cc). However, scospondin\textsuperscript{icm15/icm15} mutants retain SCO-spondin protein expression in secretory structures such as the floor plate (fp). (B, C) qRT-PCR analysis of mRNA levels of urp1 (B) and urp2 (C) in scospondin\textsuperscript{icm15/icm15} mutants (blue) compared to their control siblings (white) at 30 and 48 hpf. Data are represented as mean ± SEM. n=3 independent replicates for each condition. Each point represents a single experimental replicate. ns p>0.05, * p<0.05, ** p<0.01 (paired t-test).

We took advantage of two previously-generated scospondin alleles (Cantaut-Belarif et al., 2018) to evaluate transcriptional modifications associated with the curled-down phenotype due to the loss of the Reissner fiber in the CSF. While the scospondin\textsuperscript{icm13} null allele leads to a dual loss of the fiber in the central canal and of the SCO-spondin protein detection in secretory structures, the scospondin\textsuperscript{icm15} hypomorphic allele retains protein expression but solely precludes the Reissner fiber formation (Figure 1A). Both homozygous mutants lack the Reissner fiber and exhibit a typical curled-down phenotype, which arises from 30 hours post-fertilization (hpf) onwards ((Cantaut-Belarif et al., 2018), Figure 1A). We performed pairwise comparisons of the transcriptomes of homozygous mutants versus control siblings at 48 hpf when the body axis curvature defect is fully penetrant (Cantaut-Belarif et al., 2018). The resulting lists of up- and down-regulated transcripts that were common to the two scospondin alleles are presented in Table Supplement 1. Very few genes exhibited an important change in expression, and only a handful changed more than two folds. Noticeably, we observed a strong reduction of urp2 gene expression in curled-down mutant embryos lacking the Reissner fiber compared to their control siblings (mean ± SEM fold decrease: 4.07 ± 1.2 in scospondin\textsuperscript{icm13/icm13} and 4.69 ± 1.77 in scospondin\textsuperscript{icm15/icm15}, n=3 replicates each, p-value < 0.00005, GLM test; see Material and Methods for details). There was no significant decrease in urp1 transcript levels (mean ± SEM fold decrease: 0.97 ± 0.06
in \textit{scospondin}^{icm13/icm13} and 1.22 ± 0.25 in \textit{scospondin}^{icm15/icm15}, n=3 replicates each, p-value = p=0.191, GLM test).

The gene \textit{urp2} encodes for a secreted neuropeptide belonging to the Urotensin-II-related-peptide family (Tostivint et al., 2014). Together with \textit{urp1}, these transcripts have recently been identified as both strongly downregulated in curled-down mutants (Lu et al., 2020; Zhang et al., 2018). To ascertain our RNAseq results and confirm the difference we observed with previous results, we carried out qRT-PCR analysis of \textit{urp1} and \textit{urp2} expression levels in the hypomorphic \textit{scospondin}^{icm15} allele. Interestingly, we observed that \textit{urp1} expression level is not significantly decreased in \textit{scospondin} homozygous mutants compared to their control siblings, neither at 30 hpf nor at 48 hpf (Figure 1B). Consistently with transcriptomic results, \textit{urp2} expression level shows a strong decrease at 48 hpf in mutant embryos compared to their control siblings (3.6 ± 0.2 fold decrease; mean ±SEM; Figure 1C). This is also true at the onset of the curled-down phenotype (30 hpf: 4.2 ± 0.5 fold decrease; mean ±SEM; Figure 1C) indicating that \textit{urp2} gene expression level is affected when embryos start to develop an abnormal morphogenesis of the posterior axis. Taken together, these data show that in zebrafish embryo the presence of the Reissner fiber in the CSF is required for the normal expression level of \textit{urp2}, but not \textit{urp1}.

\textbf{The Reissner fiber is required for calcium signaling in \textit{urp2} expressing CSF-cNs}

The expression of Urotensin-II-related peptides is restricted to the ventral population of CSF-cNs (Quan et al., 2015), known to exhibit spontaneous intracellular calcium variations around 30 hpf (Sternberg et al., 2018) when the curled-down phenotype becomes visible in \textit{scospondin} mutants (Cantaut-Belarif 2018). Curled-down cilia-defective embryos lack these early calcium transients (Sternberg et al., 2018) and do not form a proper Reissner fiber (Cantaut-Belarif et al., 2018). We therefore hypothesized that the Reissner fiber may functionally interact with ventral CSF-cNs that are expressing \textit{urp2}.

To address this question, we performed \textit{in vivo} population calcium imaging at 28-30 hpf using the \textit{Tg(pkd2l1:GCaMP5G)} line labeling the dorso-lateral and ventral CSF-cNs in the spinal cord (Figure 2A, Video 1).
Figure 2. The Reissner fiber is required for the spontaneous calcium variations of ventral CSF-contacting neurons.

(A) 28-30 hpf embryos expressing the GCaMP5G calcium reporter in CSF-contacting neurons were imaged on the lateral side. *Tg(pkdl21:GCaMP5G)* embryos label both dorso-lateral (dl, above the dotted line) and ventral (v, below the dotted line) CSF-contacting neurons (arrowheads). Scale bar: 30 µm. (B) Representative traces of calcium variations in individual ventral CSF-contacting neurons in wild type (*scospondin*/*+*), heterozygous (*scospondin*icm15/*+*) and *scospondin*icm15/icm15 mutants. Sample traces from individual cells with integral ΔF/F values ranging around the median distribution of the imaged population are represented for each genotype (n=11). (C) Quantification of the normalized integrated calcium variation over time of ventral CSF-contacting neurons in wild type (+/+), heterozygous (icm15/*+*) and *scospondin*icm15/icm15 mutants (icm15/icm15, blue). Data were collected from 5 independent experiments and include 10 wild type embryos (n=146 cells), 20 heterozygous
embryos (n=287 cells) and 21 scospondin^{icm15/icm15} mutants (n=307 cells). Each point represents a single cell. Bottom and top edges of the boxes indicate the 1st and 3rd quartiles. Bold lines represent the median value for each distribution. ns p>0.05, *** p<0.001 (Kolmogorov-Smirnov test). (D) Immunohistochemistry for Pkd2l1 (magenta) and GFP (blue) in Tg(pkd2l1:GCaMP5G) embryos at 30 hpf in the spinal cord of a control sibling (left) and scospondin^{icm15/icm15} mutant (right). Scale bar: 30 µm. Magnification of the area delineated by dotted line boxes is represented for each condition (r: rostral, c: caudal, d: dorsal, v: ventral). Scale bar: 10 µm. scospondin^{icm15/icm15} embryos show a similar localization of the Pkd2l1 protein at the developing apical extension (arrowheads) of the CSF-cNs (labeled by the GFP antibody, blue) compared to control siblings. (E) In vivo voltage-clamp recordings from CSF-contacting neurons in the Tg(pkd2l1:GAL4;UAS:mCherry) line at 30 hpf in control embryos (left) and scospondin^{icm15/icm15} mutants (right). Note the extensive number of events in both conditions (top traces). Bottom traces represent higher temporal magnifications and allow distinguishing single channel openings.

See also Figure Supplement 1 and Video 1.

As previously described, we observed that ventral CSF-cNs exhibit spontaneous calcium transients in wild type embryos (scospondin^{+/+}, Figure 2B, Video 1). Quantification of the integrated fluorescence variations over time showed that in heterozygous scospondin^{icm15/icm15} embryos, which display a straight body axis and form a proper Reissner fiber (Cantaut-Belarif et al., 2018), ventral CSF-cNs retained the same level of activity than in wild type (Figure 2C). On the contrary, homozygous scospondin^{icm15/icm15} embryos exhibited a 52.8% decrease of calcium activity compared to wild type (Figure 2C). A similar reduction of 39% occurred in the null scospondin^{icm13/icm13} mutant compared to wild type siblings (Figure Supplement 1A, 1B). These data show that the loss of the Reissner fiber reduces spontaneous calcium variations of ventral CSF-cNs during the critical time window for body axis straightening. On the contrary, the sparse spontaneous calcium transients in dorsal CSF-cNs were not affected by the absence of the Reissner fiber in both scospondin alleles (Figure Supplement 1C, 1D).

Early calcium transients in CSF-cNs require the Pkd2l1 channel in vivo (Sternberg et al., 2018). The reduction in calcium activity when the Reissner fiber is lacking could therefore be due to a defect in the Pkd2l1 channel localization or opening probability. In toto immunohistochemistry for Pkd2l1 protein showed that this channel is enriched at the differentiating apical extension of CSF-cNs in curled-down scospondin^{icm15/icm15} mutants as well as in control embryos (Figure 2D). In order to assess Pkd2l1 channel properties, we performed in vivo whole cell voltage clamp recordings in double transgenic Tg(pkd2l1:GAL4;UAS:mCherry) embryos. We observed spontaneous Pkd2l1 channel openings in scospondin^{icm15/icm15} mutants similar to that observed in control embryos (Figure 2E). Thus, the loss of the Reissner fiber decreases CSF-cNs intracellular calcium variations.
without affecting the intrinsic opening probability nor the localization of Pkd2l1 channels at the differentiating apical extension of the cells. Altogether, these data suggest that the Reissner fiber is required for a signal acting on ventral CSF-cNs that controls both the spontaneous calcium variations and the expression of urp2 in the embryo.

urp2 expression is Pkd2l1-independent and compensates the loss of Reissner fiber for straightening the posterior axis

As the Reissner fiber is required for both ventral CSF-cNs spontaneous calcium transients and urp2 gene expression, we tested whether intracellular calcium variations are necessary for a normal urp2 expression and axis straightness. We took advantage of the pkd2l1icm02/icm02 mutant where the embryonic activity of CSF-cNs is abolished (Sternberg et al., 2018).
**Figure 3.** urp2 expression is Pkd2l1-independent and is important for the Reissner fiber-dependent straightening of the embryonic posterior axis.

(A) Adult wild type and pkd2l1icm02/icm02 siblings were incrossed to generate related clutches that were analyzed at 30 hpf. (B) Representative pictures of wild type (left) and pkd2l1icm02/icm02 embryos at 30 hpf (right). Note that mutant embryos develop a straight posterior axis. Scale bar: 0.5 mm. (C) Representative immunohistochemistry for the Reissner fiber a pkd2l1icm02/icm02 embryos. Note that he Reissner fiber forms properly in the central canal (cc) of the spinal cord. Scale bar: 10 µm. (D) qRT-PCR analysis of mRNA levels of pkd2l1, urp2 and urp1 in wild type (white) and pkd2l1icm02/icm02 embryos (grey). Data are represented as mean +/- SEM. n=2 independent replicates for each condition. Each point represents a single experimental replicate. ns p>0.05, * p<0.05 (paired t-test).

(E) Representative pictures of scospondinicm15/icm15 mutant embryos at 48 hpf after one cell stage injections of a control mRNA alone or of a mix containing a control mRNA and urp2 mRNA (middle and bottom). Note that upon control injections, scospondinicm15/icm15 mutants display a typical curled-down phenotype, while urp2 overexpression can lead to straightened (middle) or slightly curled-up posterior axis (bottom). Scale bar: 0.5 mm. (F) Quantification at 48 hpf of curled-down frequency in embryos obtained from scospondinicm15/+ incrosses upon control mRNA injections (n=70 curled-down animals out of 268) or urp2 mRNA overexpression (26 curled-down embryos out of 242). Data were collected from 4 independent clutches and represented as mean ±SEM. ** p<0.01 (paired t-test). (G) Injected embryos were genotyped at 48 hpf based on the loss of a restriction site in the scospondin mutant allele leading to a band resistant to digestion (−/−). While mutant animals are exclusively curled-down in control conditions, urp2 mRNA overexpression leads to the detection of mutant animals displaying a straight body axis (blue arrow).

See also Figure Supplement 2.

Thanks to the viability of pkd2l1icm02/icm02 zygotic mutants, we generated genetically-related clutches that were either fully wild type or fully maternal & zygotic (MZ) homozygous mutant (Figure 3A). We confirmed that pkd2l1icm02/icm02 MZ mutant embryos did not display any defect in body axis morphogenesis and were morphologically undistinguishable from wild type embryos (Figure 3B). Using immunohistochemistry, we observed that pkd2l1icm02/icm02 mutants form a normal Reissner fiber in the central canal of the spinal cord at 30 hpf (Figure 3C). Next, using qRT-PCR, we tested whether urp1 and urp2 gene expression levels were diminished by the absence of calcium transients in CSF-cNs (Figure 3D). As the mutation in the icm02 allele generates a premature stop codon in the pkd2l1 gene (Böhm et al., 2016), one can predict that the pkd2l1 mRNA would be degraded by nonsense-mediated decay. Indeed, mutant clutches displayed a 3.4 folds decrease in pkd2l1 transcripts level compared to wild type counterparts (Figure 3D). Interestingly, we observed that urp2 gene expression was not decreased in pkd2l1icm02/icm02 embryos compared to wild types, as well as urp1 expression that also showed no decrease (if anything, it showed a non-significant increase). Altogether, these data show that the loss Pkd2l1-driven calcium transients in CSF-cNs does
not lead to a decreased expression of urp1 and urp2 transcripts, rejecting the hypothesis of a
direct need for urp2 expression by Pkd2l1-dependent intracellular calcium variation. Instead,
our results show a strict correlation between the presence of the Reissner fiber, a normal
urp2 expression level and the proper morphogenesis of the embryonic body axis. Altogether,
our results prompted us to hypothesize that the aberrant posterior axis curvature in absence
of the Reissner fiber is a consequence of the decreased urp2 expression.

If so, restoring urp2 expression levels should rescue the posterior axis curvature
developed by scospondinicm15/icm15 mutants. To test this hypothesis, we performed one cell
stage urp2 mRNA injections on clutches obtained from scospondinicm15/+ parents. We sorted
the injected embryos at 48 hpf into two morphological categories: curled-down and non-
curled-down (Figure 3E, 3F). In the four independent experiments conducted, injections with
a control mRNA led to a proportion of curled-down embryos close to 25%, as expected
(Figure 3F). On the contrary, embryos overexpressing urp2 showed much lower proportions
of curled-down embryos (10.7 ± 1.5%; mean ±SEM; Figure 3F), suggesting that some
homozygous mutants are rescued in this condition. To confirm this rescue, we genotyped
curled-down and non-curled-down 48 hpf embryos injected with a control mRNA or urp2
mRNA (Figure 3E, 3G, Figure Supplement 2). As expected, wild type and heterozygous
embryos injected with the control mRNA displayed a straight body axis, while
scospondinicm15/icm15 showed a downward curvature of the posterior axis. Instead, urp2
overexpression lead to detect straight or slightly curled-up scospondinicm15/icm15 embryos at
several instances (Figure 3E, 3G, Figure Supplement 2). Thus, restoring higher urp2 levels is
sufficient to prevent the embryonic posterior axis defects in scospondin mutants. These
observations confirm that urp2 neuropeptide can signal at long range to ensure a proper axis
morphogenesis.

Epinephrine and norepinephrine restore morphogenesis of the posterior axis and urp
expression in scospondin mutants

Based on our observations, we assumed that the Reissner fiber is necessary for the
activity of at least one signaling pathway regulating urp2 gene expression together with body
axis morphogenesis. We investigated the possible role of epinephrine and norepinephrine in
the regulation of this signaling pathway. Both molecules belong to the monoamine
neurotransmitter family and are known to bind the Reissner fiber in rats (Caprile et al., 2003)
and frogs (Diederent et al., 1983). Recently, systemic bath applications of monoamines have
been described to rescue body axis defects in curled-down cilia defective mutants (Zhang et
al., 2018).
Thus, we tested if epinephrine and norepinephrine could influence the curled-down phenotype developed by scospondin\textsuperscript{icm15/icm15} mutants. We compared sibling animals generated from incrosses of scospondin\textsuperscript{icm15/+}.

\textbf{Figure 4. Epinephrine and norepinephrine compensate the loss of the Reissner fiber for body axis straightening and increase \textit{urp} expression.}

\textbf{(A)} Curled-down scospondin\textsuperscript{icm15/icm15} mutants and their control siblings were sorted at 30 hpf according to the geometry of their posterior axis and then exposed to a E3 solution (vehicle), epinephrine or norepinephrine for 2.5 hours prior to phenotype scoring and RNA extraction. \textbf{(B)} Representative pictures of control siblings (top) and scospondin\textsuperscript{icm15/icm15} mutants (bottom) after vehicle (left), epinephrine (middle) or norepinephrine (right) treatments. For each condition, the global morphologies of treated embryos are represented by superimposed traces linking the center of the eye, the ear and the tip of the tail in one representative clutch. Scale bar: 0.5 mm. \textbf{(C)} Quantification of the angle formed between the ear, the caudal limit of the yolk extension and the tip of the tail in control
siblings (top) and scospondin<sup>icm15/icm15</sup> mutants (bottom). Data were collected from 3 independent
experiments and include 24, 20, 25 control siblings treated with a vehicle solution, epinephrine,
norepinephrine respectively (black, solid pink and dotted pink line respectively) and 20, 24, 22
socospondin<sup>icm15/icm15</sup> embryos treated with a vehicle solution, epinephrine, norepinephrine respectively (blue, solid pink and dotted pink line respectively) *** p<0.001 (Kolmogorov-Smirnov test). (D, E) qRT-PCR analysis of the mRNA level of urp1 (D) and urp2 (E) in control siblings (left) and
socospondin<sup>icm15/icm15</sup> embryos (right). Data are represented as mean ± SEM. n=4 to 6 independent replicates for each condition. Each point represents a single experimental replicate. ns p>0.05, ** p<0.01 (GLM test).

We first analyzed the effect of 2.5 hours bath applications of epinephrine and
norepinephrine on control embryos at 30 hpf (Figure 4A). Epinephrine and norepinephrine
have a moderate impact on the shape of the head-to-tail axis of initially straight embryos,
which display a slight curled-up phenotype after exposure to monoamines (Figure 4B). To estimate the straightness of the posterior axis, we quantified the angle formed between the ear, the caudal limit of the yolk extension and the tip of the tail. This angle is distributed around 190.6° in control embryos exposed to a vehicle solution (median value; Figure 4C).

Comparatively, control embryos exposed to monoamines exhibit a distribution of ear-to-tail angles shifted towards slightly higher values (median values: 220.4° and 213.6° for epinephrine and norepinephrine, respectively; Figure 4C). Next, we analyzed the effect of epinephrine and norepinephrine on scospondin<sup>icm15/icm15</sup> embryos. While mutant animals exposed to a vehicle solution display a typical curled-down body axis, scospondin<sup>icm15/icm15</sup> mutants treated with monoamines exhibit a reduction in the downward curvature of the posterior axis (Figure 4B). Quantifications of ear-to-tail angles in scospondin<sup>icm15/icm15</sup> mutants show a large increase of the median angle value after exposure to monoamines, compared to embryos treated with a vehicle solution (106.5° for vehicle, 167.1° for epinephrine and 158.5° for norepinephrine; median values; Figure 4C). Altogether, these data show that epinephrine and norepinephrine can partially restore the posterior axis geometry of scospondin<sup>icm15/icm15</sup> mutants, suggesting that monoamines can rescue the Reissner fiber-dependent signal required for a straight embryonic body axis.

Next, we analyzed the effect of epinephrine and norepinephrine on urp1 and urp2
gene expression using qRT-PCR. In control siblings, urp1 expression remains comparable after vehicle, epinephrine or norepinephrine exposure (Figure 4D). Consistently with our previous results, urp1 expression is not significantly modified in curled-down
socospondin<sup>icm15/icm15</sup> embryos compared to their control siblings receiving the same treatment (1.01 +/- 0.17 fold change; mean ± SEM; Figure 4D). However, we observed a slight increase of urp1 expression in curled-down mutant embryos treated with epinephrine and
norepinephrine compared to vehicle treatment (1.58 ± 0.43 and 1.28 ± 0.23 fold changes for epinephrine and norepinephrine respectively; mean ± SEM). As expected, urp2 expression level is significantly decreased in curled-down *scospondin*\textsuperscript{icm15/icm15} mutants compared to straight siblings (0.22 ± 0.06 fold change; mean ± SEM; Figure 4E). Similarly to what we observed for *urp1*, epinephrine and norepinephrine treatments do not change urp2 expression in control siblings, but significantly increase it in curled-down homozygous mutant embryos (7.42 ± 4.23 and 4.67 ± 1.15 fold changes for epinephrine and norepinephrine respectively; mean ±SEM; Figure 4E).

These observations show that the rescue of the posterior axis curvature of *scospondin* homozygous mutant embryos by monoamines is associated with an increase of the expression of *urp1* and *urp2* neuropeptides. Epinephrine and norepinephrine compensate the loss of the Reissner fiber both on Urotensin-II-related neuropeptides expression and on posterior axis curvature, suggesting that these compounds act on the Reissner fiber-dependent signaling pathway in the embryonic CSF.

**Epinephrine and norepinephrine restore the Reissner fiber-dependent calcium signaling in ventral CSF-cNs of *scospondin* mutants**

The Reissner fiber is required for three concomitant events: intracellular calcium variations and *urp2* expression in ventral CSF-cNs, and the straightening of the posterior axis. We therefore asked whether the delivery of monoamines in CSF could also restore calcium variations in ventral CSF-cNs. To address this question, we performed hindbrain ventricle injections of epinephrine or norepinephrine in the Tg(*pkd2l1:GCaMP5G; scospondin*\textsuperscript{icm15}) line at 30 hpf, and recorded calcium variations in ventral CSF-cNs within the spinal cord (Figure 5A).
Figure 5. Local monoamine delivery restores calcium variations of ventral CSF-contacting neurons in scospondin mutants.

(A) Tg(pkdk2i:GCaMP5G) embryos we used to perform hindbrain ventricle injections at 30 hpf of artificial CSF (vehicle), epinephrine or norepinephrine (left). Intracellular calcium variations in ventral CSF-contacting neurons (v CSF-cNs, arrowhead) were recorded in the spinal cord 30 minutes after the injection to allow monoamines (pink dots) diffusing down the central can where bathes the Reissner fiber (RF) in control embryos. (B, C) Representative traces of calcium variations of individual v CSF-cNs in control siblings and scospondin mutants. (D) Comparison of calcium variations in control siblings versus scospondin mutants under control conditions (vehicle) and after exposure to epinephrine and norepinephrine. (E) Statistical analysis of calcium variations in ventral CSF-cNs, showing significant differences (***) between treatment groups. (F-G) DAPI-stained images of control sibling (G) and scospondin mutant embryos (G) at 30 hpf, showing expression of anti-GFP and anti-norepinephrine antibodies.
ventral CSF-contacting neurons in control siblings (B) and scospondin\textsuperscript{icm15/icm15} mutants (C) after vehicle (left), epinephrine (middle) and norepinephrine injections (right). Sample traces from individual cells with integral ΔF/F values ranging around the median distribution of the imaged population are represented for each condition (n=11). (D, E) Quantification of the normalized integrated calcium variation over time of ventral CSF-contacting neurons in control siblings (D) and scospondin\textsuperscript{icm15/icm15} mutants (E). Data were collected from 3 independent experiments and include 9, 11 and 12 control embryos recorded after vehicle, epinephrine and norepinephrine injections respectively (n=131, 150 and 164 cells respectively) and 11, 10 and 10 scospondin\textsuperscript{icm15/icm15} mutants after vehicle, epinephrine and norepinephrine injections respectively (n=168, 124 and 150 cells respectively). Each point represents a single cell. Bottom and top edges of the boxes indicate the 1\textsuperscript{st} and 3\textsuperscript{rd} quartiles. Dotted lines represent the distribution range around the 1\textsuperscript{st} and 3\textsuperscript{rd} quartiles of control embryos injected with a vehicle solution. Bold lines represent the median value for each distribution. ns p>0.05, *** p<0.001 (Kolmogorov-Smirnov test). (F) Immunohistochemistry for GFP (top) and norepinephrine (bottom) in a Tg(scospondin-GFP) embryo at 30 hpf imaged in the sagittal plane in a region of the spinal cord located above the yolk extension (representative example out of 10 embryos). Note that norepinephrine positive patterns are similar to those observed for GFP-positive material in the lumen of the central canal (cc, empty arrowheads) as well as in the floor plate (fp, plain arrowheads). Inset: magnification of the region boxed in pink. (G) Immunohistochemistry for the adrenergic receptor Adrb2 in a 30 hpf control sibling (top, representative example out of 8 embryos) and a scospondin\textsuperscript{icm15/icm15} embryo (bottom, representative example out of 9 embryos). Adrb2 is distributed in the ventral most region of the neural tube (nt) at the interface with the central canal (cc). nc: notochord. (F, G) Scale bars: 10 µm. Embryos are oriented rostral to the left and dorsal to the top. See also Video 2.

We first analyzed the effect of epinephrine and norepinephrine injections on control embryos displaying a straight body axis at 30 hpf (Figure 5B, 5D) and observed that an exogenous delivery of monoamines did not influence the basal calcium variations of ventral CSF-cNs. Next, we analyzed the effect of epinephrine and norepinephrine in scospondin\textsuperscript{icm15/icm15} mutants (Figure 5C, 5E, Video 2). When receiving vehicle injections, ventral CSF-cNs displayed a 44.1% decrease of calcium variations in scospondin\textsuperscript{icm15/icm15} embryos compared to control siblings in the same condition (median value; Figure 5D, 5E), confirming the observations reported in Figure 3. Interestingly, injections of epinephrine or norepinephrine in scospondin\textsuperscript{icm15/icm15} mutants increased the median [ΔF/F min\textsuperscript{-1}] by 70.02% and 88.6% respectively compared to vehicle injections (Figure 5E). Moreover, the rescue of the spontaneous activity of CSF-cNs by monoamines in mutant embryos reached comparable levels to those observed in control siblings (Figure 5D-E). These data show that epinephrine and norepinephrine restore the Reissner fiber-dependent calcium transients in ventral CSF-cNs in scospondin mutants. Altogether, our results are compatible with the existence of a signal that links the Reissner fiber to both urp2 expression and calcium
variation in ventral CSF-cNs that can be modulated by monoaminergic activation. These observations suggest that endogenous epinephrine and norepinephrine may act locally to tune calcium signaling in CSF-cNs.

To assess that this modulation takes place at the interface between CSF and the cells lining the central canal, we performed immunostainings against norepinephrine at 30 hpf in Tg(scospondin-GFP) embryos labeling in vivo the Reissner fiber (Troutwine et al., 2019). As previously described, SCO-spondin positive material distributes mainly into CSF to form a fiber, but punctated material packed closely to the fiber and in the floorplate is also detected (Troutwine et al., 2019; Figure 5F). Interestingly, we observed that norepinephrine positive signals follow similar patterns of distribution than densely packed material labelled by the Tg(scospondin-GFP) transgene (Figure 5F). This observation suggests that norepinephrine is a ligand endogenously present in the embryonic CSF that is associated with the Reissner-positive material in the CSF within the central canal. To address the question of the receptor associated to this signal, we performed immunostainings against the adrenergic receptor Adrb2 that is described to be transiently expressed in the zebrafish nervous system at early stages of embryonic morphogenesis (Wang et al., 2009). We observed a signal that was localized ventrally in the neural tube in a pattern that suggests a membrane location in both control siblings and scospondinicm15/icm15 mutants (Figure 5G). Thus, Adrb2 localization is suitable for binding endogenous ligands in the CSF. Altogether, these results indicate that endogenous adrenergic signals could modulate the Reissner fiber-dependent signaling pathway that instructs body axis straightening during embryonic development.
**Discussion**

Using a combination of transcriptomic analyses together with in vivo calcium imaging and pharmacology, we show here that the Reissner fiber is essential for signaling to the developing CSF-contacting neurons (CSF-cNs). In these neurons, the Reissner fiber is required for both urp2 expression and spontaneous intracellular calcium variations. This functional interaction between the Reissner fiber and ventral CSF-cNs is required for a normal curvature of the developing posterior axis of zebrafish. Using monoamine injections into the CSF, we show that the signal from the Reissner fiber to CSF-cNs can be modulated by local adrenergic activation in vivo, suggesting that the Reissner fiber acts by controlling the availability of a chemical signal in the CSF.

**urp2 expression in ventral CSF-cNs depends on the presence of the Reissner fiber and impacts on the curvature of embryonic axis**

Results in both cilia-defective and scospondin mutants suggested that mutants that fail to form a Reissner fiber display a strong downregulation of both Urotensin-II-related neuropeptides 1 and 2 in the embryo (Lu et al., 2020; Rose et al., 2020; Zhang et al., 2018). Our results contradict these observations. In the present study, our transcriptomic analysis of scospondin mutants yielded surprisingly few candidates commonly misregulated in the two alleles, and urp2 was the only gene with a strong downregulation, while urp1 showed no significant change. We confirmed this result by qRT-PCR and showed again that only urp2 is strongly downregulated, while urp1 transcripts levels were not significantly affected. In line with our results, the investigation of a newly generated scospondin hypomorphic allele (Rose et al., 2020) also revealed urp2 as the major downregulated gene in homozygous mutant and did not report a urp1 downregulation.

We further show that urp2 overexpression in scospondin mutants decreases the frequency of curled-down phenotypes, confirming that urp2 expression level is involved in the control of embryonic axis curvature (Lu et al., 2020; Zhang et al., 2018). However, urp2 may not be the only determinant of embryonic axis morphogenesis downstream of the Reissner fiber, as single urp2 morpholino knockdown do not show a defective axis curvature (Zhang et al., 2018). Parallel signaling pathways that would likely act post-transcriptionally and would therefore not be detected using our transcriptomic strategy may be involved.

The discovery of a new hypomorphic allele for scospondin recently revealed an inflammatory signature induced by the loss of the Reissner fiber at the embryonic stage (Rose et al., 2020). In our work, we did not detect such a signature in our transcriptomic analysis (see Table Supplement 1). We can speculate that these differences are due to
difference in the fish genetic background or husbandry conditions. Our work therefore begs for further studies in order to decipher the molecular pathways downstream and/or in parallel to Urp2 that regulate the morphogenesis of the embryonic axis.

Intracellular calcium variations in developing ventral CSF-cNs require the Reissner fiber

In the embryo, ventral CSF-cNs are spontaneously active via the opening of the Pkd2l1 calcium channel enriched at the level of the developing apical extension of the cells (Sternberg et al., 2018). In addition to controlling urp2 gene expression in CSF-cNs, we report here that the Reissner fiber is also required for calcium signaling in urp2-expressing cells. By investigating the pkd2l1 mutant deprived of calcium signaling in embryonic CSF-cNs, our work rejects the simple explanation that the Reissner fiber controls urp2 levels and axis straightness by increasing calcium intracellular concentrations in CSF-cNs. One question remaining is how the Reissner Fiber controls calcium variations in ventral CSF-cNs. We recently showed that the Reissner fiber is functionally coupled to the mechanosensory function of these interoceptive neurons in the larva (Orts-Del'Immagine et al., 2020). However, embryonic CSF-cNs are not fully differentiated, as they do not harbor a fully developed apical extension known to tune their mechanosensory function at larval stage (Desban et al., 2019). We therefore favor the hypothesis that the Reissner fiber acts via the modulation of the CSF content, which is supported by the restoration of calcium signaling in CSF-cNs upon monoamine injections in the brain ventricles.

Adrenergic activation restores the Reissner fiber-dependent signaling and axis straightening

Earlier reports suggest that the Reissner fiber can interact with different neuro-modulators, including monoamines (Caprile et al., 2003; Diederen et al., 1983). Monoamines unspecifically supplied in the fish water were also found to rescue body curvature in Reissner fiber-defective mutants (Lu et al., 2020; Zhang et al., 2018). They are therefore good candidates to influence the Reissner fiber signal towards ventral CSF-cNs. Our data show that the action of epinephrine and norepinephrine can compensate locally, directly in the ventricular cavities, for the loss of the Reissner fiber on calcium signaling. These two monoamines rescue the scospondin phenotype for three features: spontaneous calcium variations and Urotensin-II-related peptides expression in CSF-cNs, and body axis curvature.

One possible interpretation of our results is that the Reissner fiber is essential for the control of endogenous epinephrine and norepinephrine distribution in the embryonic CSF.
This hypothesis is supported by the presence of noradrenergic neurons in the embryonic hindbrain as early as 24 hpf (Holzschuh et al., 2003), providing a potential source of monoamines that would need to be transported caudally to the central canal of the spinal cord. This is reinforced by our results showing the localization of the Adrb2 receptor at the interface with CSF in the ventral part of the neural tube, ideally located to bind these ligands that we found closely distributed to Reissner-positive material. Interestingly Adrb2 belongs to the G protein coupled receptors family and was identified to trigger cytoplasmic calcium raise in vitro (Galaz-Montoya et al., 2017). In zebrafish, the adrenergic system plays important roles in the control of wakefulness (Singh et al., 2015) and of cardiac contractions (Steele et al., 2011). However, no morphological defects have been reported in animal missing the rate-limiting enzyme for the synthesis of epinephrine and norepinephrine (Singh et al., 2015). This might reflect the masking of the phenotype due to transcriptional adaptation to the genetic mutation (Rossi et al., 2015). Alternatively, redundant signaling pathways might mask the role of monoamines in axis curvature control. Nonetheless, our results support the idea that the cross-talk between the Reissner fiber and undifferentiated CSF-cNs is likely to be of chemical nature, possibly through monoamines themselves. Future investigations will allow to fully delineate the contribution of endogenous monoamines on ventral CSF-cNs signaling and body axis curvature, and whether they act through a direct or indirect mechanism.

Altogether, our study unravels a signal from the Reissner fiber to the developing CSF-contacting neurons. We also show that adrenergic activation can modulate this signal during embryonic body axis morphogenesis. Interestingly, a temporally controlled inactivation of cilia motility leads to spine curves reminiscent of adolescent idiopathic scoliosis (Grimes et al., 2016). Recent results suggest that, as in the embryo, the Reissner fiber and Urp reception in slow muscles are also implicated in the maintenance of a straight spine during postembryonic development (Lu et al., 2020; Rose et al., 2020; Troutwine et al., 2019; Zhang et al., 2018). Our work will pave the avenue for future investigations to identify the potential of the interplay between CSF-cNs and the adrenergic system to modulate the Reissner fiber-depend morphogenesis of the spine in the juvenile.
## Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers |
|-----------------------------------|-------------|---------------------|-------------|
| Genetic reagent (D. rerio)        | scospondin icm13 | (Cantaut-Belarif et al., 2018) | ZFIN : ZDB-ALT-181113-3 |
| Genetic reagent (D. rerio)        | scospondin icm15 | (Cantaut-Belarif et al., 2018) | ZFIN: ZDB-ALT-181113-4 |
| Genetic reagent (D. rerio)        | pkd2l1 icm02  | (Böhm et al., 2016) | ZFIN : ZDB-ALT-160119-6 |
| Genetic reagent (D. rerio)        | Tg(pkd2l1:GCaMP5G) icm02Tg | (Böhm et al., 2016) | ZFIN: ZDB-ALT-160119-4 |
| Genetic reagent (D. rerio)        | Tg(pkd2l1:GAL4) icm10Tg  | (Fidelin et al., 2015) | ZFIN: ZBD-ALT-150324-1 |
| Genetic reagent (D. rerio)        | Tg(UAS:mCherry) | (Robles et al., 2014) | ZFIN: ZBD-ALT-130702-1 |
| Genetic reagent (D. rerio)        | Tg(scospondin-GFP) | (Troutwine et al., 2019) |  |
| Recombinant DNA reagent           | pCS2-upr2    | G. Pézeron, this work |  |
| Recombinant DNA reagent           | pCS2-Ras-eGFP | (Ségalen et al., 2010) |  |
| Oligonucleotide                   | URP2_BamHI_F gcgcccGGATCCgtatctgtagaatctgctttgctgc | This work |  |
| Oligonucleotide                   | URP2_Xbal_R gcgcgcTCTAGAgccagaggctcagctgttat | This work |  |
| Oligonucleotide                   | urp2 forward: CACCGGATCCACCATCATTACC | This work |  |
| Oligonucleotide                   | urp2 reverse: GATGCCACCGCTGTCTATAGTG | This work |  |
| Oligonucleotide                   | urp1 forward: TGCGCTGCTCTGTATTCAG | This work |  |
| Oligonucleotide                   | urp1 reverse: TTTGTGCTCTTCAACCTCTG | This work |  |
| Oligonucleotide                   | pkd2l1 forward: GCACAACACCAATGAGG | This work |  |
| Oligonucleotide                   | pkd2l1 reverse: TCTCAAAGCTGTTCACCCA | This work |  |
| **Oligonucleotide** | **Lsm12b forward:** GAGACTCCTCCTCCTCTAGCAT | This work |
|---------------------|---------------------------------------------|-----------|
| **Oligonucleotide** | **Lsm12b reverse:** GATTGCATAGGCTTGGGACAAC | This work |
| **Antibody** | Anti-Reissner fiber, rabbit | (Didier et al., 1995) | Courtesy of S. Gobron |
| **Antibody** | Anti-GFP, chicken | Abcam | RRID:AB_300798 |
| **Antibody** | Anti-Pkd2l1, rabbit, | (Sternberg et al., 2018) | |
| **Antibody** | Anti-Norepinephrine, rabbit | Millipore | RRID:AB_90481 |
| **Antibody** | Anti-Adrb2, rabbit | ThermoFischer Scientific | RRID:AB_2787652 |
| **Antibody** | Alexa Fluor-488 goat anti chicken IgG | Molecular Probes | RRID:AB_142924 |
| **Antibody** | Alexa Fluor-568 goat anti-rabbit IgG | Molecular Probes | RRID:AB_10563566 |
| **Antibody** | Alexa Fluor-488 donkey anti rabbit IgG | Molecular Probes | RRID:AB_2535792 |
| **Chemical compound, drug** | DL-Norepinephrine hydrochloride | Sigma | Cat# A7256 |
| **Chemical compound, drug** | +- Epinephrine hydrochloride | Sigma | Cat# E4642 |
| **Chemical compound, drug** | MS 222 | Sigma | Cat# E10521 |
| **Chemical compound, drug** | alpha-bungarotoxin | Tocris | Cat# 2133 |
| **Software** | MATLAB | MathWorks | RRID:SCR_001622 |
| **Software** | Prism | GraphPad | RRID:SCR_002798 |
| **Software** | Fiji | (Schindelin et al., 2012) | RRID:SCR_002285 |

### Animal husbandry and genotyping

All procedures were performed on zebrafish embryos between 30 and 48 hpf in accordance with the European Communities Council Directive (2010/63/EU) and French law (87/848) and approved by the Brain and Spine Institute (Institut du Cerveau et de la Moelle épinière). All experiments were performed on *Danio rerio* embryos of AB, Tüpfel long fin (TL) and nacre background. Animals were raised at 28.5°C under a 14/10 light/dark cycle until the start of the experiment. Genotyping was performed as previously described for *scospondin<sup>icm13</sup>* and *scospondin<sup>icm15</sup>* (Cantaut-Belarif et al., 2018) and *pkd2l1<sup>icm02</sup>* (Böhm et al., 2016).
RNA sample preparation

For each experiment, condition (developmental time point and pharmacological treatment) and phenotype (either straight or curled-down), 30 sibling embryos were sorted in independent vials. After euthanasia in 0.2% MS 222 (Sigma, E10521), embryos were resuspended in 1 mL of Trizol™ (ThermoFischer Scientific, 15596026) and dissociated by multiple aspirations through the needle of a syringe. 200 µL chloroform were added prior to centrifugation and extraction of the aqueous phase. Nucleic acids were precipitated using 700 µL isopropanol, and the pellet was resuspended in 200 µL water. RNAs were purified using the RNeasy Micro Kit (Qiagen, 74004), following the provider's instruction. We performed the optional on-column DNAse treatment to improve RNAs purity. RNAs were eluted in 30 µL water to ensure a high concentration, and the quality and quantity of the extract was evaluated on a TapeStation System® (Agilent).

RNA sequencing analysis

The SmartSeq v4 (Clontech/Takara) was used to generate double-stranded cDNA from each replicate; these pools were fragmented and tagged for sequencing using the Nextera DNA Library Preparation Kit (Illumina). Finally, the prepared library was sequenced on an Illumina NextSeq 500. Quality of raw data was evaluated with FastQC (Andrews et al., 2010). Poor quality sequences was trimmed or removed using Trimmomatic software (Bolger et al., 2014) to retain only good quality paired reads. Star v2.5.3a (Dobin et al., 2013) was used to align reads on GRCz11 reference genome using standard options. Quantification of gene and isoform abundances was acheived using with Rsem 1.2.28, prior to normalization with the edgeR bioconductor package (Robinson et al., 2009). Finally, differential analysis was conducted with the GLM framework likelihood ratio test from edgeR.

The quality control and PCA analysis (not shown) indicated that one sample was not reaching the same reproducibility as the two others, even after trimming and normalization. We therefore used the average value of the two most reliable replicates to calculate the average expression level (count per million, cpm) of all the 28 214 genes either in straight controls or in curled-down embryos (average of the expression in the two alleles icm13 and icm15). We then filtered out low-expression genes that had an expression level below 1 cpm in the average of the straight control replicates. We then kept genes that had an average fold change between curled embryos and controls of 0.75 for down-regulated genes (120 genes), and 1.45 for up-regulated genes (94 genes). We turned back to the original raw cpm values of the three replicates in the two alleles for these two short-lists. We tested the consistency of
the fold change across replicates and across alleles with a general linear model (GLM) (Nelder et al., 1972). The design matrix included 2 regressors of interest (encoding whether the sample is a straight control or curled embryos) and 2 confounding variables (the unwanted variability that might be associated with the two different genetic environments of the two families of fish carrying the icm13 and the icm15 allele). Statistical significance of the effect of interest (above and beyond confounding factors) was tested using a t-test. Because of the potential false discovery associated with multiple testing, we then used the Benjamini–Hochberg procedure (Benjamini, et al., 1995).

Potential genes of interest were sorted according to their increasing p-values. To shorten the list of potential genes of interest, we determined at which rank i the p-value became higher than the Benjamini–Hochberg criterion using \((i/\text{total nb})^* 0.2\). This method allows for up to 20% of false-positives, but avoids the rejection of true-positives that was manifest with a more stringent correction as Boniferri. For example, the scospondin mRNA level in the icm13 allele is highly decreased, presumably through non-sense mediated decay: the Boniferri correction would have rejected this result as not significant, whereas the Benjamini–Hochberg procedure keeps it in the list of potentially interesting genes. However, this procedure requires a post-hoc validation with an independent technique for each potential gene of interest, as we did by qRT-PCR (see below).

Quantitative RT-PCR

1.2 µg of each RNA sample was retro-transcribed using the Verso cDNA Synthesis Kit (ThermoFischer Scientific, AB1453B) following the provider’s instructions. A 1:3 ratio of random hexamer:polydT primers was used to favor mRNA amplification without a strong bias for the 3’ end of messengers. qPCR experiments were performed using the LightCycler® 480 SYBR Green I Master kit (Roche, 04707516001) on a LightCycler® 96 machine (Roche). Each pair of primers (see Key Resource Table) was tested beforehand on a given cDNA stock that was diluted in a series of 4 points. Linearity of CT variation with the cDNA dilution as well as single peaks in the melting curves corresponding to single amplicons were assessed. For each new RNA extraction, we tested that no amplification was detectable when the PCR was performed directly on the RNA stock (-RT). We used the housekeeping gene lsm12b as an internal reference in each experiment. qPCR were repeated two to three times for each cDNA stock and the average CT was used for further calculations. The relative abundance of the gene of interest was evaluated using the CT comparison formula: \(2^{-\Delta\text{CT}}\). All results were obtained on at least three biological replicates (except for pdk2l1 mutant extracts), each originating from a single mating.
Pharmacology and quantification of body axis curvature

30 hpf embryos from *scospondin*\(^{icm15/+}\) incrosses were treated during 2.5 hours with E3 medium alone (vehicle), epinephrine hydrochloride (Sigma, E4642) or norepinephrine hydrochloride (Sigma, A7256) both diluted to 3 mM in E3 medium. To ensure a proper quantification of ear-to-tail angles, embryos were then fixed over-night in 4% PFA 6-well plates, rinsed in 1X PBS, mounted laterally in 1.5% low-melting point agarose using a AZ100M macroscope (Nikon). For each embryo, the angle between the ear, the caudal limit of the yolk extension and the tip of the tail was quantified using Fiji (Schindelin et al., 2012). Representative traces of the global morphology of the embryos after treatment were drawn for all experimental conditions on one representative clutch by linking the center of the eye to the ear and following the dorsal line linking somite boundaries until the tip of the tail.

In vivo calcium imaging

*\(Tg(pkd2l1:GCaMP5G)\) embryos were manually dechorionated at 30 hpf, mounted laterally in 1.5% low-melting point agarose, and paralyzed by injecting 1-2 nL of 500 µM alpha-bungarotoxin (Tocris, 2133) in the caudal muscles of the trunk. When required, hindbrain ventricle injections were performed using artificial cerebrospinal fluid (aCSF, containing in mM: 134 NaCl, 2.9 KCl, 1.2 MgCl\(_2\), 10 HEPES, 10 glucose and 2.1 CaCl\(_2\); 290 mOsm.kg\(^{-1}\), adjusted to pH 7.7–7.8 with NaOH) as a vehicle solution. Epinephrine hydrochloride (Sigma, E4642) and norepinephrine hydrochloride (Sigma, A7256) were diluted to 3 mM in aCSF before brain injections. Calcium imaging was performed at 4 Hz using a spinning disk confocal microscope (Intelligent Imaging Systems, Denver) for 4 minutes. Imaging was restricted to the region of the spinal cord beyond the yolk extension. Regions of interest were manually selected based on an average projection of the time-lapse and according to the dorso-ventral position of the cells. The integrals of normalized \(\Delta F/F\) signals were calculated using a custom script on MATLAB (see (Sternberg et al., 2018)).

In vivo patch clamp recording

Whole-cell recordings were performed in aCSF on 30 dpf *\(Tg(pkd2l1:GAL4;UAS:mCherry)\) embryos carrying the *scospondin*\(^{icm15/+}\) mutation and their respective control siblings. Embryos were pinned through the notochord with 0.025mm diameter tungsten pins. Skin and muscle from two to three segments around segment 10 were dissected out using a glass suction pipette. A MultiClamp 700B amplifier, a Digidata series 1440 A Digitizer, and pClamp 10.3 software (Axon Instruments, Molecular Devices, San Jose, California, USA) were used for acquisition. Raw signals were acquired at 50 kHz and low-pass filtered at 10 kHz. Patch
pipettes (1B150F-4, WPI) with a tip resistance of 5–8MΩ were filled with internal solution containing in mM: K-gluconate 115, KCl 15, MgCl2 2, Mg-ATP 4, HEPES-free acid 10, EGTA 5 or 10, 290 mOsm/L, pH adjusted to 7.2 with KOH with Alexa 488 at 40 μM final concentration). Holding potential was −85 mV, away from the calculated chloride reversal potential (E_Cl = -51 mV).

**Immunohistochemistry and confocal imaging**

Embryos were manually dechorionated and euthanized using 0.2% MS 222 (Sigma, E10521) prior to fixation. Samples were fixed 2 hours in 4% PFA-3% sucrose, except for norepinephrine stainings where embryos were fixed in 1.5% PFA-1.5% glutaraldehyde. The yolks and the skin from the rostral part of the trunk were removed and embryos were blocked overnight in a solution containing 0.5% Triton, 1% DMSO and 10% normal goat serum (NGS). Primary antibodies were incubated at 4°C in a solution containing 0.5% Triton, 1% DMSO and 1% NGS. All secondary antibodies were from Molecular Probes© and used at 1:500. The following dilutions of primary antibodies were used: rabbit anti-Reissner fiber 1:200 (Didier et al., 1995), rabbit anti-Pkd2l1 1:200 (Sternberg et al., 2018), chicken anti-GFP 1:500 (Abcam ab139170), rabbit anti-norepinephrine 1:100 (Millipore, AB120), rabbit anti-Adrb2 1:200 (ThermoFischer Scientific, PA5-80323). Systematic omission of the primary antibody confirmed the specificity of the immunostaining results. Zebrafish embryos were mounted laterally in Vectashield® Antifade Mounting Medium (Clinisciences, H1000) and imaged on an inverted SP8 DLS confocal microscope (Leica). Images were then processed using Fiji (Schindelin et al., 2012). Maximal Z-projections on 6-9 microns in depth are represented.

**RNA microinjections**

To produce mRNA, urp2 CDS was amplified from cDNA by PCR and cloned (BamHI - Xbal) into pCS2+. Messenger RNA were produced with the mMESSAGE mMACHINE™ kit (Ambion™). 1 nL RNA-containing solution was injected into 1- to 2-cell stage embryos obtained from scospondin^icm5^ incrosses. Each clutch was separated into three groups: uninjected and injected either with a control mRNA (100 ng/μL, ras-eGFP encoding for a membrane tagged GFP, (Ségalen et al., 2010) or with a mix containing control mRNA and urp2 mRNA (100 ng/μL total, 1:1 ratio). To assess for injection quality, GFP-positive embryos were first sorted out at 1 dpf and then scored at 48 hpf for body axis curvature defects.

**Statistics**
All values are represented as boxplots (median ± interquartile range) or mean ± SEM (stated for each in the figure legend). All statistics were performed using MATLAB and Excel. In the figure panels, asterisks denote the statistical significance calculated using the appropriate test (stated for each test in the legends): *, p<0.05; **, p<0.01; ***, p<0.001; ns, p>0.05.
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Author contributions

Y.C.B.: conceptualization, data curation, formal and statistical analysis, investigation, methodology. M.P: data curation. A.O.D.: data curation. G.P.: methodology. P.L.B: conceptualization, data curation, statistical analysis, supervision, funding acquisition, validation. C.W.: conceptualization, supervision, funding acquisition, validation.

Competing interests

Claire Wyart: reviewing editor, eLife. Other authors declare no competing interests.

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Table Supplement 1. Up- and down-regulated transcripts in curled-down *scospondin* mutants compared to their control siblings.

After filtering for low expression, we kept a list of genes that were differentially expressed in null *scospondin*^{icm13/icm13} and hypomorphic *scospondin*^{icm15/icm15} mutant embryos at 48 hpf. The table shows the average fold changes in the two alleles and their mean. Genes are ranked based on their p-value that compare the reproducibility of the change inside and between the two alleles using a GLM framework. We adjusted the false discovery rate due to multiple comparisons using the Benjamini-Hochberg procedure (see Material and Methods for details).
Figure Supplement 1. The spontaneous activity of dorso-lateral CSF-contacting neurons does not require the Reissner fiber in the embryo.

(A) Traces of calcium variations in ventral CSF-contacting neurons at 30 hpf in wild type (scospondin\textsuperscript{+/+}), heterozygous (scospondin\textsuperscript{icm13/+}) and scospondin\textsuperscript{icm13/icm13} null mutants. Sample traces from individual cells with integral $\Delta F/F$ values ranging around the median distribution of the imaged population are represented for each genotype (n=11). (B) Quantification of the normalized integrated calcium variation over time of ventral CSF-contacting neurons in wild type (+/+) heterozygous (icm13/+), and scospondin\textsuperscript{icm13/icm13} mutants (icm13/icm13, blue). Data were collected from 3 independent experiments and include 8 wild type embryos (n=107 cells), 13 heterozygous embryos (n=176 cells) and 22 scospondin\textsuperscript{icm15/icm15} mutants (n=277 cells). Each point represents a single cell. Bottom and top edges of the boxes indicate the 1\textsuperscript{st} and 3\textsuperscript{rd} quartiles. Bold lines represent the median value for each distribution. ns $p>0.05$, *** $p<0.001$ (Kolmogorov-Smirnov test). (C, D) Quantification of the normalized integrated calcium variations over time of dorsolateral CSF-contacting neurons in 30 hpf embryos obtained from scospondin\textsuperscript{icm15/+} incrosses (C) and scospondin\textsuperscript{icm13/+} incrosses (D) n=95, 243 and 255 cells in wild type, heterozygous and
spondin}\textsuperscript{icm15/icm15} embryos respectively (C); n=141, 176 and 168 cells in wild type, heterozygous and spondin}\textsuperscript{icm13/icm13} embryos respectively (D). ns p>0.05 (Kolmogorov-Smirnov test).
Video 1. Intracellular calcium transients of ventral CSF-contacting neurons are reduced in scospondin mutants.

Sagittal views of the spinal cord of Tg(pkd2l1:GCaMP5G) embryos at 30 hpf. scospondin+/+ (top), scospondin icm15/+ (middle) and scospondin icm15/icm15 siblings (bottom) are represented. In wild type and heterozygous embryos, ventral CSF-contacting neurons are more active that dorso-lateral ones. Note the sharp decrease in calcium variations of ventral CSF contacting neurons in the homozygous mutant embryo compared to wild type and heterozygous counterparts. Data were collected at 4 Hz and displayed at 80 Hz. Scale bar: 30 µm.
Figure Supplement 2. *urp2* overexpression recues body axis curvature defects in *scospondin* mutants.

Summary of straight, curled-up and curled-down phenotypes detected in wild type (+/+) and *scospondin* ^icm15/+ (+/−) and *scospondin* ^icm15/icm15^ mutant embryos (−/−) at 48 hpf after one cell stage injection of control mRNA (A) or control +*urp2* mRNA (B). Note that 2 mutant (−/−) embryos were detected as straight and 2 as curled-up upon *urp2* overexpression. Data were collected over 3 independent clutches.
Video 2. Epinephrine and norepinephrine restore intracellular calcium transients of ventral CSF-contacting neurons in scospondin mutants.

Sagittal views of the spinal cord of Tg(pkd2l1:GCaMP5G) embryos at 30 hpf in a straight control sibling (top) and curled-down scospondin^{icm15/icm15} mutants after vehicle, epinephrine or norepinephrine injections in the hindbrain ventricle. Note that calcium transients are restored in a subset of ventral CSF-contacting neurons of scospondin^{icm15/icm15} embryos that received epinephrine and norepinephrine injections compared to mutants that received a vehicle injection. Data were collected at 4 Hz and displayed at 80 Hz. Scale bar: 30 µm.