A right-handed signalling pathway drives heart looping in vertebrates

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Most animals show external bilateral symmetry, which hinders the observation of multiple internal left–right (L/R) asymmetries that are fundamental to organ packaging and function1,2. In vertebrates, left identity is mediated by the left-specific Nodal–Pitx2 axis that is repressed on the right-hand side by the epithelial–mesenchymal transition (EMT) inducer Snail1 (refs 3, 4). Despite some existing evidence5,6, it remains unclear whether an equivalent instructive pathway provides right-hand-specific information to the embryo. Here we show that, in zebrafish, BMP mediates the L/R asymmetric activation of another EMT inducer, Prrx1a, in the lateral plate mesoderm with higher levels on the right. Prrx1a drives L/R differential cell movements towards the midline, leading to a leftward displacement of the cardiac posterior pole through an actomyosin-dependent mechanism. Downregulation of Prrx1a prevents heart looping and leads to mesocardia. Two parallel and mutually repressed pathways, respectively driven by Nodal and BMP on the left and right lateral plate mesoderm, converge on the asymmetric activation of the transcription factors Pitx2 and Prrx1, which integrate left and right information to govern heart morphogenesis. This mechanism is conserved in the chicken embryo, and in the mouse Snail1 acts in a similar manner to Prrx1a in zebrafish and PRRX1 in the chick. Thus, a differential L/R EMT produces asymmetric cell movements and forces, more prominent from the right, that drive heart laterality in vertebrates.

Defects in L/R asymmetry occur in 1:10,000 humans7, and the associated morbidity and mortality usually indicate congenital heart defects8,9. The EMT converts epithelial cells into migratory cells, and it is required in tissues and organs that are generated after profound cell movements, such as the mesoderm and the neural crest9. Thus, deregulation of L/R asymmetries and EMT leads to severe congenital malformations or early embryonic lethality1,9.

After an initial disruption in L/R symmetry in the vertebrate embryo, laterality is conferred to the organizer (node) and this information is transferred to the left lateral plate mesoderm (LPM)1. The left-specific programme in the LPM is driven by Nodal and its downstream target Pitx2. This pathway is conserved in deuterostomes, and in chick and mouse embryos it is repressed on the right-hand side by the EMT-inducer Snail1 (refs 3, 4). We have found that like Snail1 in amphibians, a prrx1 gene duplicate (prrx1a), is transiently expressed in the LPM of the zebrafish embryo with higher levels on the right (Fig. 1a). Prrx1a, like Pitx2, is a paired-like homeobox transcription factor, and like Snail1, is an EMT inducer in embryos and cancer cells10.

In vertebrates, the convergence of the left and right cardiogenic regions in the embryonic midline results in the formation of a linear primary heart tube (PHT). The subsequent bilateral addition of progenitor cells to the arterial (anterior) and venous (posterior) poles from the corresponding second heart fields (SHFs), respectively contribute to the elongation and growth of the heart at the outflow and inflow tracts11. These additions occur at the time of heart looping, for which the underlying mechanisms remain poorly understood11, although defects in the posterior pole are responsible for numerous congenital heart defects in humans. Considering Prrx1a L/R asymmetric expression and its described role in the induction of cell movements10, we examined whether prrx1a knockdown influenced heart position. Both prrx1a morpholino oligonucleotide (prrx1aMO1 or prrx1aMO2)10 injections or CRISPR–Cas9-driven prrx1a mutations led to mesocardia, a straight heart that failed to undergo the normal dextral looping (Fig. 1b, c and Extended Data Fig. 1). Both atrial and ventricular chambers are specified in the morphants (Fig. 1d), but, in addition to defective looping, the heart presented a smaller atrium and a defective posterior pole lacking a defined sinus venosus and the expression of its marker Isl1 (refs 12, 13) (Fig. 1e and Extended Data Fig. 2a–c). However, the anterior pole was not overly affected (Extended Data Fig. 2d).

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Figure 1 | prrx1a L/R asymmetric expression in zebrafish. a, Whole-mount in situ hybridization and transverse sections at the levels of the dotted lines in 14-, 18- and 24-somite stage zebrafish embryos. b, Heart location in control and prrx1a-morphant (prrx1aMO2) embryos analysed for the cardiomyocyte marker myl7. hpf, hours post fertilization; MO, morpholino. c, Quantification of heart location. d, Analysis of atrial (A) and ventricular (V) markers amhc (also known as myh6) and vmhc, respectively. e, Confocal analysis of Prrx1 and Isl1 expression in transgenic zebrafish embryos Tg(tbSa::eGFP). Nuclei were stained with DAPI. Asterisks indicate areas of asymmetry. Scale bars, 50 µm or 10 µm (enlarged images in e)
Because Tbx5a is a transcription factor expressed in PHT and in LPM cells that contribute to the pericardium and the posterior pole,14,15 we generated a tbx5a-reporter transgenic line Tg(tbx5a:eGFP) that allowed us to see its coexpression with Prrx1a in the precursors of the posterior pole once the PHT had been formed (Extended Data Fig. 3) and to follow the movements and fate of this population of Prrx1a+ cells. Cell-tracing experiments in this area using the photoconvertible green-to-red Kaede protein indicated that Prrx1a+ cells contributed to both the pericardium and the atrium, explaining the defective atrium in the prrx1a morphants (Fig. 2a). These atrial cells do not express Prrx1 any longer, indicating that prrx1a is downregulated after incorporation into the heart. As such, Prrx1 protein was never observed in the heart tube, which was positive for tbx5a (Fig. 2a and Extended Data Fig. 4a–d). Notably, other embryonic and cancer cells also downregulate EMT transcription factors, including PRRX1, when these cells lose their mesenchymal nature and undergo differentiation upon reaching their final location.8

We also followed the cell movements towards the posterior pole and found that they were defective in the morphants concomitantly with the failure of heart looping (Fig. 2b and Supplementary Videos 1, 2). We detected actomyosin stress fibres in Prrx1+ cells, which also had an elongated cell shape and elongated nuclei. These phenotypic traits, when associated with cell movements, are characteristic of mesenchymal cells undergoing EMT (Fig. 2b, c), which were absent in morphant embryos. In turn, morphants showed numerous tbx5a+ cells that failed to contribute to the posterior pole and the atrium, remaining dispersed throughout the pericardium (Extended Data Fig. 5a), again explaining their defective posterior pole (Fig. 1e and Extended Data Fig. 2a–c). Altogether, this indicates that Prrx1a might direct Tbx5a+ cardiac progenitors to the posterior pole in a bilaterally asymmetric manner, with a greater contribution from the right.

Directed cell migration has already been implicated in heart jogging, an initial leftward displacement of the PHT driven by Nodal in zebrafish.16,17 Although we found randomization of heart jogging in prrx1a morphants (Extended Data Fig. 5b, c), the majority of embryos developed mesocardia regardless of the direction of jogging (Supplementary Videos 3, 4), indicating that the Prrx1-mediated driving force for heart looping occurs at later stages, compatible with looping being independent of both jogging and the Nodal pathway.16,18

The asymmetric contribution of right and left cells may generate asymmetric forces and greater tension on the right-hand side, inducing a maintained leftward displacement of the posterior pole that would promote the dextral torsion of the heart19–21, the two steps in looping morphogenesis. Compatible with this, the tbx5a reporter revealed cells that appeared to form a structure reminiscent of a cable (Fig. 2d). To assess the contribution of asymmetric tension to heart looping, we laser ablated a small population of Tbx5a+ Prrx1a+ LPM cells adjacent to the PHT on either side after jogging and before looping. Heart lateralization was only affected after ablating cells in the right LPM (Fig. 2e and Extended Data Fig. 6a–c). In vivo visualization of actin stress fibres and actin and myosin double labelling confirmed the formation of a cable during looping, a structure that was absent in prrx1a morphants (Fig. 2f, Extended Data Fig. 6d, e, and Supplementary Videos 5–8).

We next found that palladin (Palld), an actin-binding protein that promotes actin bundling in migratory and invasive cells22,23, was coexpressed with Prrx1 and Tbx5a in the cable-like structure, and downregulated in the prrx1a morphants (Fig. 2g), linking Prrx1 with the cytoskeleton. This actomyosin cable is reminiscent of others that also control forces during embryonic development22,25. Its function during looping is compatible with the mesocardia found when zebrafish PHT explants18 or embryos were exposed to the myosin inhibitor blebbistatin (Extended Data Fig. 6f and Supplementary Videos 9, 10) and when chick embryos were treated with cytochalasin to inhibit actin polymerization26, suggesting that this mechanism may be conserved in the chick.

A transient asymmetric distribution of PRRXI was evident in the chick LPM, with higher levels on the right-hand side (Fig. 3a) at stages in which SNAIL1 (also known as SNAI1) is also asymmetrically expressed (Extended Data Fig. 7a and ref. 27). PRRXI was also asymmetrically expressed in the sinus venosus horns (Fig. 3b). As in the zebrafish, PRRXI is not expressed in the cardiac crescent or PHT, but it is expressed by the population of TBX5+ cells that are lateral and posterior to the cardiac posterior pole (Extended Data Fig. 7b–d). Bilateral downregulation of PRRXI in the chick embryo induced mesocardia in most embryos (Fig. 3c and Extended Data Fig. 7e, f). However, PRRXI unilateral downregulation on the left half did not affect heart looping (Fig. 3d and Extended Data Fig. 7g), whereas overexpression could induce reverse looping (Fig. 3e and Extended Data Fig. 7h), further evidence that PRRXI L/R asymmetric expression drives heart laterality.

As in the zebrafish embryo, deficiency in PRRXI expression did not prevent the specification of atrial and ventricular cell fates, the size of the atrium was reduced and did not seem to affect the anterior pole (see outflow tract) (Extended Data Fig. 7i–k). Similarly, ISLET1 colocalized with PRRXI in the posterior pole and its expression was downregulated.
with a Nodal inhibitor increased PRRX1 levels (Fig. 4c), as has previously been shown for SNAIL1 (ref. 30). Noggin can still decrease PRRX1 levels in the presence of the Nodal inhibitor (Fig. 4c), suggesting that the BMP pathway controls PRRX1 expression directly. As such, BMP4 beads on the left side reversed both PRRX1 L/R asymmetric expression and heart looping (Fig. 4a and Extended Data Fig. 8h). The PRRX1 gene contains two consensus-binding sites (P and D) for the BMP-downstream effectors SMAD1/SMAD5/SMAD8 (Extended Data Fig. 8i), and SMAD5 binds to both binding sites (Extended Data Fig. 8j), compatible with its proposed role in heart looping in the mouse31. The activity at the PRRX1 promoter increases upon BMP4 administration only when these binding sites are intact (Extended Data Fig. 8k). Thus, BMP signalling directly activates PRRX1 gene transcription.

Incubation of whole zebrafish embryos with BMP4 or Noggin, respectively increased or decreased prrx1a expression, and Nodal inhibition abolished prrx1a L/R asymmetry by derepressing Prrx1a on the left-hand side of the embryo (Fig. 4d). Hence, BMP-mediated prrx1 expression is stronger on the right-hand side to drive normal heart looping, as this right pathway is downregulated on the left-hand side by Nodal both in zebrafish and chick embryos. Thus, two parallel and mutually repressed pathways driven by two TGFβ superfamily members (Nodal and BMP), activate paired-like homeobox transcription factors, Pitx2 and Prrx1, respectively, integrating left–right information (Fig. 4e).

As SNAIL1 has also been implicated in heart laterality1,3, we examined PRRX1 and SNAIL1 in the chick and both are expressed in the sinus venosus horns with higher levels on the right, covering mutually exclusive territories in the lateral mesoderm (Fig. 5a and Extended Data Figs 9a, 10a). SNAIL1 downregulation with SNAIL1MO reproduced the effects described3, provoking mesocardia in 26.4% of embryos and L-loop in 15.7% of embryos (Extended Data Fig. 9b–d). Simultaneous downregulation of SNAIL1 and PRRX1 induced mesocardia (50% of embryos) with a distribution similar to that found after PRRX1 downregulation (Extended Data Figs 7f, 9b–d). Hence, SNAIL1 contributes to heart positioning in the chick acting independently from PRRX1, the downregulation of which induces mesocardia independently from SNAIL1 function.

In the zebrafish embryo, neither snail1a nor snail1b, which are the result of a teleost whole-genome duplication25, showed asymmetric L/R expression (Extended Data Figs 9e and 10a), therefore these genes probably do not regulate heart positioning. This was confirmed in morphant embryos (Extended Data Fig. 9g–i). This lack of L/R asymmetry also suggests that, unlike in chick and mouse3,4, Snail1 may

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**Figure 3** PRRX1 also drives heart looping in the chick embryo. 

a, b, PRRX1 expression in whole chicken embryos and transverse sections. SH; sinus venosus horn; SoLM, somatic lateral mesoderm; SpLM, splanchnic lateral mesoderm; SV, sinus venosus. c, Bilateral (B) electroporation with an eGFP-reporter construct plus PRRX1 or control siRNA. d, e, Unilateral electroporation (left, L) with control or PRRX1 siRNA (d) or with a vector containing the PRRX1-coding sequence (e). f, Immunofluorescence of ISLET1 and PRRX1 in control and PRRX1-siRNA-electroporated embryos. AIP; anterior intestinal portal; LSH/RSH; left/right sinus venosus horn. g, PALLD expression in similarly treated embryos. Asterisks, areas of asymmetry. Nuclei were stained with DAPI (blue). Dotted lines indicate the level of the sections shown. Scale bars, 250μm or 50μm (sections in f, g).

in embryos electroporated with PRRX1 short interfering RNA (siRNA) (Fig. 3f and Extended Data Fig. 7l). This population of sinus venosus progenitors expressing TBX18 (Extended Data Fig. 8a), contributes to the posterior pole together with the SHF, and has been referred to as posterior SHF20,28 or tertiary heart field29, because it is located more laterally than the classical NKX2.5+ SHF. Notably, TBX18 is repressed when PRRX1 is downregulated (Extended Data Fig. 8b). Actin fibres were observed in a cable-like structure of PRRX1+ cells at the wall of the right vitelline vein and right sinus venosus horns in control embryos (Extended Data Fig. 8c), compatible with the displacement of the posterior pole to the left side. The loss of phalloidin staining and L/R morphological asymmetry after PRRX1 silencing (Extended Data Fig. 8c) explains the mesocardia observed. The role of actomyosin is confirmed by the mesocardia that occurred after incubation with blebbistatin (Extended Data Fig. 8d). As in the zebrafish, PRRX1 downregulation also decreased PALLD expression, which was coexpressed with PRRX1 in a L/R asymmetric manner in control embryos (Fig. 3g and Extended Data Fig. 8e, f).

We next investigated the signals that induce PRRX1 expression and how PRRX1 is integrated into the pathways involved in L/R asymmetry. BMP induces PRRX1 and SNAIL1 expression in the chick embryo16, and the latter represses PITX2 transcription on the right-hand side3,4. Administration of BMP4 on the left or its inhibitor, Noggin, on the right LPM, respectively induced or repressed PRRX1 expression (Fig. 4a). Ectopic NODAL expression on the right downregulated PRRX1 expression (Fig. 4b and Extended Data Fig. 8g), and incubation...
control of heart positioning operates from zebrafish to mice (Fig. 5g and Extended Data Fig. 10c). In the mouse, SNA1L1 not only represses left-handed information on the right\textsuperscript{1,4}, but also instructs the right-handed pathway leading to heart looping. Similarly, Prrxl\textsubscript{a} achieves both roles in the zebrafish (Extended Data Fig. 10d). In summary, while a well-described left-hand side Nodal–Pitx2 pathway promotes and stabilizes left identity\textsuperscript{1}, a BMP pathway operates prominently on the right not only to repress the Nodal–Pitx2 pathway, but also to provide instructive information that drives heart looping and morphogenesis.

While the Nodal–Pitx2 axis is not conserved in worms and flies, the BMP–Prrx1 pathway activates actomyosin, and myosin acts as a L/R determinant in Drosophila\textsuperscript{36}, suggesting a conserved role in laterality during bilaterian evolution. Whether the unilateral leftward cell movement that breaks bilateral symmetry in the early chick embryo is also driven by a similar mechanism remains to be investigated further\textsuperscript{37}.

Heart looping is essential for both atrial–ventricular and heart–vasculature concordance\textsuperscript{2}, and therefore, for proper organ function. Defining the events underlying this process may shed light on the evolution of the heart from the simple straight invertebrate tube acting as a peristaltic pump to a suction pump in zebrafish and a rhythmically beating structure in amniotes. These data should also help to better understand the congenital heart defects derived from disrupted heart laterality in humans.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions O.H.O. performed the majority of the experiments, analysed the data and contributed to writing. H.C. performed histological and expression studies. M.G. generated the anti-Prrx1 antibody in E.M.T.’s laboratory, and J.G. designed and performed the CRISPR-mediated prrx1a-promoter analysymd. M.A.N. acknowledges financial support from the AEI through the ‘Severo Ochoa’ Programme for Centres of Excellence in R&D (SEV-2013-0317) to Instituto de Neurociencias (IN).

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**Methods**

**Embryos.** Zebrafish strains AB wild-type, Tg(actb:2-myl12.1-mCherry) to visualize non-cardiac myosin II (ref. 38) and Tg(tbxa:aeGFP) (see below) were maintained at 28 °C under standard conditions, and the embryos were staged as described previously39. Fertilized hen eggs were purchased from Granja Gilbert (Tarragona, Spain) and incubated in a humidified incubator at 37.5 °C. Chick embryos were staged according to Hamburger and Hamilton40 (HH) or somite number. Normal mouse embryos were obtained from natural matings of C57BL/6J mice. The age of mouse embryos was determined as days postcoitum (dpc) and by somite number. Snail1p116, Tg(UBC-crc/ERT2)Ig65, Gt(Rosa)26Sgtm1Pol4E(GAL4-tdTomato)HsJ (Snail1fl/fl) (ref. 41) ubiquitin-driven Cre/ERT2 (ref. 42), tdTomato-Cre reporter43) embryos were obtained from pregnant females that were gavage-fed 250 μl of 30 mg ml⁻¹ tamoxifen dissolved in 5% ethanol in corn oil (Sigma-Aldrich) at 6.5 dpc. Embryos were obtained at 8.5 dpc. This combined genotype circumvents the early lethality caused by the loss of function of Snail1 before gastrulation44. UBC-crc/ERT2 provides a temporal control of Cre activity, the presence of tamoxifen at 6.5 dpc allows Cre activity after gastrulation in all the cells of the embryo. Tamoxifen-induced Cre activity was monitored by the presence of tdTomato fluorescence. An additional Snail1-conditioned mutant model in which deletion was driven by the Meox-Cre line (Snail1 deleted in embryonic tissues from around 8 dpc) has been used previously4. In both conditional models, Snail1 is deleted after early gastrulation and early L/R determination, and coinciding with the time when Snail1 is transiently expressed in a L/R asymmetric manner in the LPM. Both models show heart laterality defects and our data provide the cellular and molecular mechanism that explains this phenotype.

All animal studies were performed without randomization to form the experimental groups. Sample size was not predefined for experiments involving embryos. For ChIP experiments five biological replicates were done, luciferase experiments were done on primordial embryos using four biological replicates. No blinding was used in the experimental design; however, results were independently scored by two people. No selection of sex of the animals was performed, because in all cases the embryos were younger than the sex-determination stage. All the experimental animals used here were at embryonic stage.

All animal procedures were conducted in strict compliance with the European Community Council Directive (2010/63/EU) and Spanish legislation. The protocols were approved by the CSIC Ethical Committee and the Animal Welfare Committee at the Institute of Neuroscience.

**Generation of the Tg(tbxa:aeGFP) transgenic line.** The bacterial artificial chromosome (BAC) containing the zebrafish tbxa gene (CH73-99A14) was obtained from the BACPAC Resources Center. A GFP-polyA cassette was introduced into the zebrafish tbxa-containing BAC by homologous recombination45. In brief, a 588 bp 5′ homology arm encompassing part of the zebrafish tbxa first intron, the 5′ UTR of the second exon and a 479 bp 3′ arm starting at the ATG of the tbxa gene present in its second exon, were cloned into the PL451 vector (provided by N. G. Copeland). The resulting targeting construct was electrotransfered into EL250 cells containing the CH73-99A14 BAC in order to enable homologous recombination and incorporation of the GFP-polyA cassette into the endogenous tbxa ATG contained in the BAC. Homologous recombination was confirmed by sequencing the PCR fragments obtained with primers external to the targeting construct. The primer sequences used for the construction of the targeting construct and confirmation of the homologous recombination are available upon request. The NucleoBond BAC 100 Kit (MACHEREY-NAGEL) was used to obtain BAC DNA, and 1 nl of the 70 ng BAC DNA was injected into the blastodisc of fertilized eggs, after which the embryos were allowed to develop. On reaching sexual maturity, F₂ zebrafish were individually crossed to wild-type zebrafish and eGFP reporter F₁ embryos were raised to adulthood to create the stable Tg(tbxa:aeGFP) transgenic line used here. The analysis of the Tg(tbxa:aeGFP) reporter was carried out taking into consideration our previous expression experiments46. When the reporter was combined with immunofluorescence (that is, Fig. 2g and (Extended Data Fig. 9g). The standard control morpholino from Gene Tools was used in control injections. Capped nlKaede48 and LifeAct-GFP/LifeAct-RFP49 mRNA were synthesized with the SP6 mMessage mMachine kit (Ambion), and 75 and 100 pg was injected into one-cell embryos, respectively.

**Electroporation of siRNA, morpholinos and vectors.** Chick embryo explants (stage HH3–4) were placed ventral side up on filter paper rings and electroporated with selected plasmids, siRNA or morpholinos as described previously40. Unilateral and bilateral electroporations were performed as follows. For unilateral electroporation embryos were injected with plasmid and/or siRNA into the left or right side and the sample was placed face down on filter paper rings and incubated over the samples. To electroporate the whole population of cardiomyocyte precursors (bilateral electroporation), the positive platinum electrode was placed transverse to the primitive streak after the sample injection. Electroporations were performed bilaterally except for the experiments shown in Fig. 3d, e, 4b and Extended Data Fig. 8g that were unilateral, left (L) or right (R). Gain-of-function experiments were performed by unilateral electroporation (Fig. 3e). pMn-Cndonad55, a gift from C. Stern (UCL, UK) was used at 4 μg l⁻¹ (Fig. 4b). The previously described pCX-PRRX1 vector (1 μg l⁻¹) or the pMt or pCX empty vectors (for control embryos) were combined with a pCX-eGFP plasmid (0.5 μg l⁻¹) to follow electroporation. Loss-of-function experiments were performed by unilateral or bilateral electroporation as indicated. Two siRNA against PRRX1 were designed (see Supplementary Table 2 for sequences). A mix of these siRNA constructs (2 μM each) plus the pCX-eGFP plasmid (0.5 μg l⁻¹) and 6% sucrose was injected. Control embryos were electroporated with a scrambled siRNA. To interfere with the Snail1 expression two splice-site-blocking fluorescein-labelled morpholinos were designed (Snail1MO1 and Snail1MO2, see Supplementary Table 2 for sequences) and a mix of these two morpholinos (1 μM each) was electroporated alone or in combination with the two siRNA sequences against PRRX1 and pCX-RFP as a control for electroporation. The standard control fluorescein-labelled morpholino from Gene Tools was used as a control. SNAIL1 morpholino efficiency at preventing splicing was tested by RT–PCR as described above. Oligonucleotides in exons 1 and 2 for SNAIL1MO1 that yield a fragment of 255 bp, and in exon 2 and 3 for SNAIL1MO2 that yield a fragment of 288 bp were used. The unspliced fragments are 950 and 1,244 bp respectively.

**CRISPR–Cas9.** Cas9–NLS–6×His (pET-28b-Cas9-His; Addgene, 47327 (ref. 52), was expressed in E. coli, purified on a Ni-column and stored at −20 °C in 300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol, pH 7.4 at 25 °C. For sgRNA production, oligos were obtained from Sigma-Aldrich and cloned in BsaI-digested DR274 (Addgene 42250)56. The primer sequences are listed in Supplementary Table 2. The sgRNA was synthesized using MEGAlightscript T7 (Ambion) and purified with the MEGAcleave kit (Ambion). For RNP formation and injection into one-cell zebrafish embryos, Cas9 (800 ng μl⁻¹) was incubated for 5 min at 37 °C with equimolar amounts of in vitro transcribed sgRNA in the presence of 150 mM KCl. RNPs were injected into one-cell embryos. To perform T7 endonuclease test, DNA extracted from 24 hpf embryos was used to amplify a fragment containing the target region of prrx1a. After PCR, the amplicon was denatured and allowed to anneal. Digestion was performed with T7 endonuclease 1 (New England Biolabs) according to the manufacturer’s instructions. Heart location and presence of mutans was determined in 89 embryos. The tail of each embryo was used to individually extract DNA and perform a T7 endonuclease test. The rest of the embryo was fixed and used for in situ hybridization with myl7 to determine heart location. Ratio of mesocardia to normal hearts in CRISPR-induced mutant embryos (crispants) was 54% (n = 48/89), which is very similar to that of the prrx1aMO morphants (49%, n = 80/164) (Extended Data Fig. 1d–f). Analysis of the CRISPR-induced mutations was performed by amplification of the exon 1 region and subsequent cloning and sequencing of the products. We detected mutations in all the embryos, the nature of the mutations was diverse and in all cases consistent with NHEJ repair of the resulting cuts produced around the location of the guides.

**Electroporation of siRNA, morpholinos and vectors.** Zebrafish embryos were treated from the 8- to 22-somite stage with 0.02 mg ml⁻¹ of human recombinant BMP4 protein (R&D Systems), Noggin (0.03 mg ml⁻¹; R&D Systems) or the Nodal inhibitor SB431542 (Addgene 42250)56. The primer sequences are listed in Supplementary Table 2. The embryos were treated from the 8- to 22-somite stage with 0.02 mg ml⁻¹ of human recombinant BMP4 protein (R&D Systems), Noggin (0.03 mg ml⁻¹; R&D Systems) or the Nodal inhibitor SB431542 (100 μM; Sigma-Aldrich). Embryos were also treated from 29 to 50 hpf with blebbistatin (25 μM; Sigma-Aldrich), while the control embryos received 0.1% DMSO alone. Before treatments the embryos were dechorionated to improve drug accessibility. Chick embryos were grown in easy culture as described elsewhere54 and exposed to 1 ml of SB431542 (100 μM) from the 1- to 10-somite stage or to 1 ml of blebbistatin (50 μM) from the 1- to 14-somite stage. After fixation, the embryos were processed for in situ hybridization. BMP4 (0.2 μg ml⁻¹) or Noggin (0.5 μg ml⁻¹) was applied on to the embryos for 3 h at room temperature. The beads were then placed into one side of the LPM at stage HH7 and the embryos were collected 7 h later. Beads soaked in PBS were used as controls.

**Whole-mount in situ hybridization.** Whole-mount in situ hybridization using digoxigenin-labelled probes was performed on zebrafish, chick and mouse.

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embryos. Fluorescent in situ hybridizations were performed as described. Probes against zebrafish prrx1a, crestin, lbbp, snaila, snailbl, pitx2c, chicken PRRX1 (ref. 11) and PRRX2 were described previously. Templates for zebrafish myl7, amhc, vmhc, chicken AMHC, VMHC, TBX5, TBX18, NKX2.5, PALLD, PRRX2, mouse Prrxl and Prrx2 were amplified by PCR from their respective cDNA sequences and subcloned into the pGEMT-easy vector (Promega) or pCDNA3 (Thermo Fisher) with the primers indicated in Supplementary Table 2 and used to generate probes. Some embryos were embedded in paraffin, gelatin or low-melting-temperature agarose and sectioned at 7, 20 or 150 μm, respectively. For detail about probes, see Supplementary Table 3.

Anti-Prrx1 antibody generation. To develop the rabbit polyclonal anti-Prrx1 antiserum, the N terminus of axolotl (Ambystoma mexicanum) Prrxl (amino acids 1–101) was cloned as a fusion protein with glutathione S-transferase (GST) or maltose-binding protein (MBP). The GST–Am–Prrx1-N and MBP–Am–Prrx1-N fusion proteins were expressed in E. coli (BL21-DE3) and purified using glutathione sepharose 4B (GE) and amylose resin (New England Biolab), respectively. GST–Am–Prrx1-N was injected into a rabbit to generate a polyclonal antiserum, which was affinity purified against MBP–Am–Prrx1-N immobilized on a NHS-activated sepharose 4 Fast Flow column (GE). The antiserum was dialysed and concentrated with an Amicon Ultra-15 10K MWCO (Millipore) before use.

Immunofluorescence. Whole-mount embryos were fixed with 4% PFA overnight at 4°C. After fixation, the embryos were dehydrated and rehydrated in graduated PBT (PBS and 0.1% Tween 20)–methanol series. Antigen retrieval was performed by maintaining the embryos for 15 min at 70°C in 150 mM Tris-HCl buffer (pH 9) and the embryos were permeabilized with cold acetone for 20 min at −20°C. After washing with PBT, embryos were blocked with 10% FBS for 3 h at room temperature and incubated with the primary antibodies overnight at 4°C. After washing for several hours in PBT, the embryos were incubated with the secondary antibodies (Cy3, 1:100, or Cy5, 1:100; Jackson ImmunoResearch) for 2 h. Some embryos were also embedded in gelatin or low-melting temperature agarose and sectioned at 150 μm. For immunofluorescence on sections, embryos were embedded in paraffin and sectioned at 7 μm. Immunostaining was performed by standard procedures. Antigen retrieval was performed on the paraffin sections by incubation for 20 min at 100°C in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Sections were incubated with the primary antibodies overnight at 4°C and then incubated with the secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI (4′,6-diamidino-2′-phenylindol dihydrochloride) by incubation of the samples in 1 μg/ml DAPI in PBT. Embryos were incubated overnight at 4°C and for 1 h at room temperature. Samples were washed extensively in PBT before mounting. For antibody details, see Supplementary Tables 1, 3.

Detection of actin and stress fibres. Detection of stress fibres and actin was performed using either TRITC– or FITC-conjugated phalloidin. Fixed embryos were treated with a 1:50 dilution of labelled Phalloidin in PBT at 4°C overnight. After extensive washing in PBT, embryos or sections were directly analysed on the confocal microscope. For life imaging of actin polymerization, GFP– or RFP–conjugated LifeAct was used. Zebrafish one-cell embryos were injected with mRNA encoding a GFP– or RFP–conjugated LifeAct and embryos were followed or fixed for the desired time. LifeAct was detected using an antibody against the conjugated fluorescent protein (GFP or RFP) in all cases in which the samples were previously fixed.

Seminthin sections. Zebrafish embryos were fixed overnight at 4°C with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB). The resulting nuclei were labelled with a yellow line in Extended Data Fig. 3b. The resulting nuclei were labelled with a yellow line in Extended Data Fig. 3b. Quantification of the asymmetrical expression of Prrx1a.

To identify Prrxl+ cells in the LPM we took advantage of the Tg(tbxsaczGFP) zebrafish line and the antibody against Prrx1. Transgenic embryos at the 22–somite stage were fixed and subjected to double immunofluorescence against Prrxl and GFP. Images were collected at a dorsal view and the images were analysed using the Imaris software (Bitplane AG). Each individual cell was identified as single- or double-positive for the two markers. We developed a mask using ImageJ soft ware (https://imagej.nih.gov/ij/) for all the double-positive cells (Prrxl+, GFP−). The resulting nuclei were labelled with a yellow line in Extended Data Fig. 3b. These cells were counted and heat maps of the number of double-positive cells and protein levels of Prrxl are shown in the Extended Data Fig. 3c. Both the number of cells and the levels of Prrxl protein are higher on the right-hand side.

Time-lapse imaging. For whole-embryo imaging, embryos were dechorionated, anaesthetized with 0.02% tricaine (MS222; Sigma-Aldrich) and mounted in 3% methylcellulose on a glass-bottom 35-mm culture dish (MatTek) containing an imprinted pattern of 1% agarose to provide support. Confocal imaging of the Tg(tbxsaczGFP) embryos was performed using a Spectral Confocal Microscope (Leica TCS SP2) acquiring z-stacks every 10 min (step-size of 5 μm up to 300 μm depth). During imaging, embryos were maintained in an incubation chamber heated to 28.5°C.

Kaede photoconversion. For cell-tracing experiments embryos were injected with 75 pg of in vitro transcribed mRNA encoding the photoconvertible green-to-red fluorescent protein Kaede into the one-cell stage. Embryos at 22 hpf were mounted as above for live imaging. Photoconversion of Kaede was performed unilaterally at the defined region of interest (ROI) illuminated with a diode laser at 405 nm (Radius 405 nm, 50 mW, Coherent Inc.) at 1.1 × optical zoom and 80% laser power during 10 s. z-stacks of green and red fluorescence were acquired immediately before and after photoconversion. To avoid further exposure to UV light, embryos were incubated in the dark until 50 hpf when they were imaged again.

Laser ablation. The Tg(tbxsaczGFP) embryos were mounted at 26 hpf as described for time-lapse imaging. Laser ablation was performed unilaterally with a two-photon laser (Femtosecond Mai Tai HP Ti: sapphire mode-locked laser system, Spectra Physics) integrated on a spectral confocal microscope (Leica TCS SP2 MP). Irradiation over the selected region of eGFP+ cells was performed using an optical zoom of 1.2×, laser wavelength of 800 nm and maximum laser power. Before and after laser ablation, z-stacks were taken and maximum projections were created in order to evaluate the damage of the targeted cells. Embryos were incubated at 28°C and imaged again at 50 hpf to determine the post-ablation phenotype. Embryos were fixed in 4% PFA and some of them were used to assess heart looping by in situ hybridization with the cardiac myosin light chain marker myl7.
purification. Tissue was cross-linked with 1% formaldehyde in PBS for 10 min at room temperature and subsequently quenched with 0.125 M glycine for 15 min. Embryos were washed in cool PBS and resuspended in 300 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8 and protease inhibitor cocktail), followed by 15 min incubation on ice. Lysates were sonicated three times for 15 min in a Bioruptor (Diagenode) (H, 30 s on/30 s off on ice). Sonicated lysates were diluted to 5 ml with dilution solution (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl, protease inhibitor cocktail), and used for antibody incubation. Antibodies (listed in the Supplementary Table 1) were added to the chromatin dilutions and incubated overnight with continuous rotation at 4°C. The chromatin–antibody mixtures were added to overnight-blocked Dynabeads–protein A beads (Thermo Fisher) (blocking solution is 0.05% BSA, 2 mg ml−1 salmon sperm DNA in dilution solution), and incubated for 4 h with continuous rotation at 4°C. The beads were washed in four consecutive steps with wash buffers (WB)1–4 using a magnetic concentrator (WB1: 0.1% SDS, 1% Triton X-100, 2 mM Tris-HCl pH 8 and 150 mM NaCl; WB2: 0.1% SDS, 1% Triton X-100, 2 mM Tris-HCl pH 8 and 500 mM NaCl; WB3: 1 mM EDTA, 10 mM Tris-HCl pH 8, 1% IgGpreal (NP40), 1% sodium deoxycholate and 0.25M LiCl; WB4: 10 mM Tris-HCl pH 8 and 1 mM EDTA). 1% Chelex was added to the beads, followed by vortexing and incubating at 95°C for 10 min. Proteinase K treatment (40 μg ml−1 in WB4) was performed by incubating at 55°C for 30 min with agitation followed by a 95°C incubation for 10 min with agitation. Samples were centrifuged at 13,000 r.p.m. for 2 min and the supernatant, which contains DNA, was used for qPCR assays with the primers indicated in Supplementary Table 2.

Promoter-activity assays. Distal and proximal SMAD-containing regions were cloned from chick genomic DNA using the indicated primers. PCR fragments were cloned into the pGL3–Promoter vector (Promega). Mutagenesis was performed using the QuickChange Kit (Agilent) with the primers indicated in Supplementary Table 2.

A pool of five chick embryos were collected after dissection at stage HH35 to obtain embryonic fibroblasts. Head, internal organs and bones were removed and the rest was minced with sterile scissors and digested with trypsin for 5 min at 37°C. Cells were pelleted and resuspended in DMEM supplemented with 5% FCS and 1% penicillin–streptomycin. Fibroblasts were purified by their differential attachment to plates for 1 h and subsequent washing out of the other cell types. After 24 h, 4 × 106 cells per well were seeded and transfected with 20 ng of the pRLE-TK control vector plus 100 ng of either the PRX1–promoter fragments cloned into a pGL3–promoter (Promega) or an empty vector to be used in luciferase assays. Treatment with BMP4 (10 ng ml−1) was performed 24 h after transfection and lasted for 24 h. Cell lysates were collected and firefly and renilla luciferase assays. Treatment with BMP4 (10 ng ml−1) was performed 24 h after transfection and lasted for 24 h. Cell lysates were collected and firefly and renilla luciferase luminescence assays were performed using a Dual Luciferase Assay (Promega) as described by the manufacturer.

Statistical analysis. Statistical analysis was performed using the GraphPad Prism software package. Results were expressed as mean ± s.e.m. (standard error of mean). Differences among groups were tested by using two-tailed paired or unpaired Student’s t-tests or one-way ANOVA, as indicated in the figure legends. Differences were considered statistically significant when P < 0.05 (**P < 0.05, ***P < 0.01, ****P < 0.001).

Anatomical nomenclature. We have used herein the term ‘posterior pole’ to refer to the cardiac structures that are located at the caudal end of the developing heart in all the animal models. This term is equivalent to ‘venous pole’ or ‘inflow tract’ used by other authors. In the posterior pole, cells migrating from the posterior SHF incorporate and contribute to part of the atrium and to all of the sinuses venosi, the most posterior cardiac chamber. This process occurs in zebrafish, chick and mouse. The main difference between the anatomical structures developing at the posterior pole of these models is the lack of sinus venosi horns in zebrafish embryos. In both chick and mouse, the sinus venosi comprises a central common chamber expanded at both sides by lateral horns (RSA1 and LSH) that are posteriorly continuous with the vitelline veins. The myocardial/smooth muscle transition marks the limit between the sinus venosi horns and the vitelline veins. The anterior or arterial pole of the zebrafish heart has been referred to as outflow tract, since this is the usual term in the literature. We have used the term anterior intestinal portal (AIP) for the anterior invagination of the endoderm that gives rise to the foregut. Sinus venosi horns and vitelline veins are successively located at the lateral margins of the AIP. The lateral plate mesoderm splits into somatic and splanchic lateral mesodermal layers during the formation of the coelomic cavity. The somatic lateral mesoderm (SOML) is in contact with the ectodermal cell layer. We have used the term dorsal splanchic lateral mesoderm (DSPLM) to refer to the mesodermal layer close to the invaginated foregut endoderm, and ventral splanchic lateral mesoderm (VSPLM) for the mesoderm in contact with the non-invaginated endodermal lateral to the AIP. This mesoderm contributes to the walls of the vitelline veins and the sinus venosi horns. A full list of abbreviations of anatomical structures is included in Supplementary Table 4.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.
Extended Data Figure 1 | prrx1a downregulation induces heart-looping defects. a, Downregulation of prrx1a transcripts upon prrx1aMO1 microinjection. Ctrl, control. Data represent mean ± s.e.m.; ***P < 0.001; Student’s t-test; n = 3 biological replicates. b, Microinjection of a prrx1aMO2 morpholino located in the 5′ UTR also impairs normal heart looping at 50 hpf. Heart looping assessed by in situ hybridization with a myl7 probe in embryos microinjected with control or prrx1a MO2 morpholinos. The frequency of the phenotypes is indicated below the images. c, Scheme of the prrx1a locus indicating its exon organization. The coding sequence is depicted in blue and the location of the single-guide RNAs (sgRNA) is indicated. The location of primers used for screening is indicated by the two coloured arrows. d, Representative images of ‘crispants’ obtained by injection of the indicated sgRNA and Cas9. The frequency of the phenotypes is indicated below the images. e, Representative T7 endonuclease assays of four embryos per phenotype indicated in d and two wild-type (WT) embryos used as negative controls. f, T7 endonuclease assay of 8 crispants and one WT embryo after injection of the indicated sgRNA. g, The PCR products obtained from embryos 1, 2 and 5 in f were cloned and sequenced. Representative sequences of those embryos (embryo number indicated on the right), with the mutations indicated in red and the deletions with hyphens. The location of the sgRNA is indicated in green and the corresponding adjacent PAM is highlighted in yellow. Scale bars, 100 μm.
Extended Data Figure 2 | Cardiac phenotype of prrx1a-morphant embryos. a, Bright-field images of whole-mount and haematoxylin–eosin staining of sagittal sections of control and prrx1a-morphant embryos (n = 5 embryos per condition). b, Snapshots of surface-rendering images of representative control and prrx1a-morphant embryos hybridized with the atrial marker amhc. The graph represents the area of amhc expression (μm²; n = 7 per condition). Data represent mean ± s.e.m.; ***P < 0.001; Student’s t-test. c, Parasagittal semithin sections of control and morphant embryos (48 hpf) obtained at the right (R), middle (M) and left (L) levels. The ventricle (V) is on the right and the sinus venosus (SV) is well-developed in the control. However, the morphant has the ventricle aligned with the atrium (A), and lacks a SV in the cardiac posterior pole (PP). OFT, outflow tract. d, Ventral view of a maximum-intensity projection and z-plane sections of confocal images (right) through the outflow tract showing the expression of the anterior SHF marker latent TGFβ binding protein-3 (ltp3) in control and prrx1a-morphant zebrafish embryos. The cardiomyocytes are visualized by MF20 immunofluorescence. Scale bars, 50μm.
Extended Data Figure 3 | Prrx1a expression in the early embryo.

a, Maximum-intensity projection of confocal images showing Prrx1 expression in Tg(tbx5a:eGFP) embryos with DAPI staining of cell nuclei (blue). Prrx1 and tbx5a colocalize in the LPM (left). The asymmetric L/R expression of Prrx1 in the right LPM is better seen in the single-channel view (middle) and the transverse sections at the level of the white dotted line (right). tbx5a is absent from the neural crest (NC) and the posterior LPM (PLPM). b, Prrx1 and tbx5 colocalization (yellow) identifies the Prrx1a+ cardiac precursor population once the PHT has formed. Number of double-positive cells in the left and right halves of the LPM. Data represent mean ± s.e.m.; **P < 0.01; Student’s t-test; n = 6. c, Heat map of Prrx1-expression levels in the LPM cells identified in b. Colour code is shown on the right. Intensity was measured in individual cells of the selected regions in the left and right LPM and an average intensity value was obtained per side. The plot shows the average value obtained in the right relative to the left LPM. Data represent mean ± s.e.m.; *P < 0.05; Student’s paired t-test; n = 6. d, Dorsal view of zebrafish embryos to visualize the expression of prrx1a and the pan-neural crest marker crestin. Colocalization of prrx1a and crestin identifies the prrx1a+ neural crest cell population. tbx5 is excluded from the neural crest. NT, neural tube. Scale bars, 50 μm.
Extended Data Figure 4 | Prrx1 and Tbx5 co-localization in developing heart tissues. a–c, Ventral views or sagittal sections showing more Prrx1+ Tbx5a+ cells in the visceral pericardium (P) in contact with the heart tube (HT) on the right side (arrowheads in sections and enlarged images in a and c). Prrx1 is absent from the heart tube. Boxes in c indicate the areas highlighted on the right. d, Sagittal sections of a 48 hpf zebrafish embryo showing Prrx1 expression compared with the cardiomyocyte marker MF20, or the atrial marker S46. Scale bars, 50 μm. A, atrium; E, eye; PV, pericardial vesicle; SV, sinus venosus; V, ventricle.
Extended Data Figure 5 | Cell movements during heart development in the zebrafish embryo. a, Snapshots from time-lapse analysis of Tg(tbx5:eGFP) control and prrx1 morphant embryos at heart-looping stages. b, Randomization of cardiac jogging in prrx1a morphants. Representative images of control (Ctrl MO) and prrx1a-morphant (prrx1a^{MO1}) embryos hybridized with a myosin light chain 7 (mly7) probe to assess heart tube jogging direction. Images are dorsal views with anterior to the top. Scale bars, 100 μm. c, Quantification of heart-jogging direction. A, atrium; PHT, primary heart tube; SV, sinus venosus; V, ventricle.
Extended Data Figure 6 | Photoablation of mesodermal cells in the LPM. a, b, Photoablation was performed at 26–27 hpf on the left (a) or the right side (b). Ventral views with merged bright-field and z-plane confocal images, before and after unilateral photoablation (Pa) of tbx5:eGFP+ cells. The photoablated areas are located in the most anterior region of the LPM adjacent to the limit of the extra-embryonic tissue. Higher power images of the photoablated areas (right panels) correspond to the boxed areas. c, Analysis of heart location at 50 hpf. Hearts visualized after myl7 in situ hybridization in wild-type and left- or right-photoablated embryos. Mesocardia only develops in embryos photoablated on the right-hand side. d, Cartoon of a ventral view of a zebrafish embryo at 30–32 hpf with a boxed area that corresponds to that in the enlarged areas shown in Fig. 2f, g, e, Snapshots of actomyosin fibres, generated by the merged images extracted from time-lapse confocal microscopy in live control (left) and prrx1a-morphant embryos (right) of the Tg(tbx5a:eGFP) reporter line injected with LifeAct (n = 7 embryos per condition). Brackets indicate the region of the cable-like structure. f, Snapshots from time-lapse videos of Tg(tbx5a:eGFP) embryos treated with blebbistatin or with the vehicle alone. Scale bars, 50 μm.
Extended Data Figure 7 | See next page for caption.
Extended Data Figure 7 | PRRX1 expression in chick embryos.

a, Whole-mount in situ hybridization in chick embryos showing the transient asymmetric expression of SNAIL1 in the LPM. b, Expression analysis by double fluorescence in situ hybridization of PRRX1 and NKK2.5 or TBX5 in whole-mount embryos. c, Transverse sections showing PRRX1 and NKK2.5 or TBX5 expression at the levels indicated by dotted lines in b. Boxes indicate the position of the corresponding enlarged images. The arrowhead indicates the centre of the posterior pole displaced to the left. d, Dual fluorescent in situ hybridization for PRRX1 and the atrial (AMHC) or ventricular (VMHC) myosins. High power images of transverse sections as indicated on the left showing the absence of myocardial PRRX1 expression. e, Downregulation of PRRX1 transcription after electroporation with siRNA. Data represent mean ± s.e.m.; ***P < 0.001; Student’s t-test; n = 3 biological replicates.

f–h, Quantification of heart location from experiments shown in Fig. 3c, d. RNAi, RNA interference. i, AMHC and VMHC in control embryos and in those electroporated with PRRX1 siRNA. j, Morphometric analysis of the atrium in chick embryos electroporated with control siRNA or PRRX1 siRNA. The graph represents the area of AMHC expression (μm²; n = 7 embryos per condition). Data represent mean ± s.e.m.; **P < 0.01; Student’s t-test. k, Expression of FGF8, required for outflow tract formation, is not altered by PRRX1 downregulation.

l, PRRX1 and ISLET1 coexpression in the lateral right sinus venosus horn (RSH) (arrows). Scale bars, 500 μm (a), 10 μm (enlarged images in l) or 100 μm (all other panels). A, atrium; AIP, anterior intestinal portal; CF, cardiac folds; LPM, lateral plate mesoderm; LSH/RSH, left/right sinus venosus horns; OFT, outflow tract; SH, sinus venosus horns; V, ventricle.
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | **PRRX1 is essential in the posterior pole of the heart.**

**a,** Double fluorescent *in situ* hybridization for TBX18 and PRRX1 in chick embryos. **b,** TBX18 downregulation in embryos electroporated with PRRX1 siRNA. **c,** Maximum-intensity projection of dual fluorescent staining of PRRX1 and phalloidin (a mycotoxin that stains actin stress fibres). Stars indicate the area of asymmetry and colocalization. **d,** Heart looping assessed by MF20 immunofluorescence in embryos treated with vehicle or blebbistatin. **e,** Maximum-intensity projection showing the cells coexpressing PALLD (palladin) and PRRX1. **f,** PALLD is decreased upon PRRX1 downregulation. Data represent mean ± s.e.m. of four pools (five embryos each) of control and PRRX1-knockdown embryos; *P < 0.05, **P < 0.01; Student’s *t*-test. **g,** Visualization of electroporated areas in chick embryos shown in Fig. 4b. Embryos were co-electroporated on the right side with an eGFP-reporter construct plus an empty vector or a vector containing the NODAL-coding sequence. **h,** Heart location in embryos containing control BSA- or BMP4-loaded beads. **i,** Diagram of the PRRX1 promoter. Proximal (P) and distal (D) binding sites for SMAD1/5/8 and constructs used in **j** and **k.** **j,** ChIP with antibodies against SMAD5 or IgG (negative control). Data represent mean ± s.e.m.; ***P < 0.001; ns, not significant; Student’s *t*-test; *n = 5 biological replicates. **k,** Luciferase activity of PRRX1 promoter. Both distal (D) and proximal (P) SMAD5-binding sites are functional. Fragments containing these sites can activate the promoter in primary chick fibroblasts upon BMP4 treatment. Mutations in these sites prevent the response to BMP4. Data represent mean ± s.e.m.; ***P < 0.001; ns, not significant; one-way ANOVA; *n = 4 biological replicates. Dotted lines show the level of the sections. Scale bars, 100 μm or 10 μm (enlarged images and sections in **a, b**). A, atrium; AIP, anterior intestinal portal; CF, cardiac folds; LPM, lateral plate mesoderm; LSH/RSH, left/right sinus venosus horns; OFT, outflow tract; SV, sinus venosus; V, ventricle; VV, vitelline veins.
Extended Data Figure 9 | SNAIL1/snail1 and PRRX1 in heart looping in vertebrate embryos. **a**, PRRX1 and SNAIL1 expression in whole-mount chick embryos and transverse sections of chick embryos. **b**, Maximum-intensity projection of confocal images showing heart location by triple immunofluorescence in embryos electroporated with control fluorescein-labelled morpholinos, with a mix of two fluorescein-morpholinos against SNAIL1 or a combination of SNAIL1 morpholinos plus PRRX1 RNA interference. An RFP construct was used as a reporter for electroporated cells. **c**, Schematic drawing and knockdown-efficiency assay of fluorescein-labelled morpholinos directed to SNAIL1 splicing sites. **d**, Quantitative analysis of heart location. **e**, snail1a is not expressed in the LPM in zebrafish embryos. **f**, snail1b expression in whole-mounted embryos and transverse sections at the levels of the dotted lines. **g**, Schematic drawing and knockdown efficiency assay of the snail1b splice site morpholino (snail1bMO). **h**, Quantification of heart location in control and snail1b morphants. **i**, snail1b is not expressed in the pericardium (P) in the zebrafish embryo. Scale bars, 500 μm (a, left panel), 50 μm (i) or 100 μm (all other panels).
Extended Data Figure 10 | Model for heart looping in vertebrates. a, Cartoon depicting the comparative expression of Prrx1/PRRX1 and Snail1/SNAIL1 in zebrafish, chick and mouse embryos. b, Transverse sections showing the lack of PRRX2 expression in the sinus venosus horns of chick at HH9–10 and Prrx2 expression in mouse embryos at 8.5 dpc. Scale bars, 100 μm. c, Schematic representation of the proposed model for heart looping in vertebrates in relation to the expression of prrx1a/PRRX1/Prrx1 and snail1b/SNAIL1/Snail1. d, A prominent right-handed pathway drives differential L/R EMT and heart looping in vertebrates. In addition to the left-specific Nodal–Pitx2/PITX2 pathway that confers left identity and represses the right-handed pathway, BMP signalling activates EMT in the LPM in a L/R asymmetric manner to drive heart laterality and the repression of leftward information on the right through the repression of Pitx2/2/PITX2. This conserved cellular mechanism is implemented through the activation of different EMT inducers in different vertebrates. Nomenclature in the diagrams is unified for all species for simplicity. Correct names are as in the legend. A, atrium; AIP, anterior intestinal portal; C, coelom; DSpLM, dorsal splanchnic lateral mesoderm; Ec, ectoderm; FG, foregut; LA, left atrium; LSH, left sinus venosus horn; LV, left ventricle; LVV, left vitelline vein; NC, neural crest; N, notochord; NP, neural plate; NT, neural tube; OFT, outflow tract; PV, pericardial vesicle; RA, right atrium; RSH, right sinus venosus horn; RV, right ventricle; RVV, right vitelline vein; SoLM, somatic lateral mesoderm; S, somite; SV, sinus venous; V, ventricle; VSpLM, ventral splanchnic lateral mesoderm; YS, yolk sac.