Jam1a–Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling

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Notch signalling plays a key role in the generation of haematopoietic stem cells (HSCs) during vertebrate development1–4 and requires intimate contact between signal-emitting and signal-receiving cells, although little is known regarding when, where and how these intercellular events occur. We previously reported that the somitic Notch ligands, Dc and Dld, are essential for HSC specification5. It has remained unclear, however, how these somitic requirements are connected to the later emergence of HSCs from the dorsal aorta. Here we show in zebrafish that Notch signalling establishes HSC fate as their shared vascular precursors migrate across the ventral face of the somite and that junctional adhesion molecules (JAMs) mediate this required Notch signal transduction. HSC precursors express jam1a (also known as fli1r) and migrate axially across the ventral somite, where Jam2a and the Notch ligands Dc and Dld are expressed. Despite no alteration in the expression of Notch ligand or receptor genes, loss of function of jam1a led to loss of Notch signalling and loss of HSCs. Enforced activation of Notch in shared vascular precursors rescued HSCs in jam1a or jam2a deficient embryos. Together, these results indicate that Jam1a–Jam2a interactions facilitate the transduction of requisite Notch signals from the somite to the precursors of HSCs, and that these events occur well before formation of the dorsal aorta.

JAM proteins belong to the immunoglobulin superfamily of cell adhesion molecules, comprised of three closely related members, Jam1 (also known as JAM-A or F11R), Jam2 (also known as JAM-B), and Jam3 (also known as JAM-C). It has been reported that Jam1 is expressed in both murine and zebrafish HSC fractions6, although its role in haematopoiesis is unclear. In zebrafish, Jam1a and Jam1b are expressed in bilateral stripes of posterior lateral mesoderm (PLM) (Extended Data Fig. 1a–d), which gives rise to both endothelial and haematopoietic lineages5. After 18 hpf, however, Jam1a was no longer detected in endothelial cells (Extended Data Fig. 2b, c). We performed co-staining of Jam1a with fli1, a marker of the vascular lineage. The expression domain of fli1 overlapped with that of Jam1a at 14 hpf (Extended Data Fig. 2d), indicating that PLM cells indeed express Jam1a at this stage. We observed the downregulation of Jam1a in purified green fluorescent protein (GFP)-labelled endothelial cells (fli1;GFP+ cells) from 14 to 20 hpf (Extended Data Fig. 2e).

To determine if HSC precursors are contained within Jam1a+/PLM cells, we performed lineage tracing using the combined transgenic lines, -2.2jam1a:CreERT2, which expresses CreERT2 under the control of jam1a regulatory elements, and bactin2loxP-BFP-loxP-DsRed, which switches from expression of blue fluorescent protein (BFP) to the DsRed red fluorescent protein following Cre-based recombination (Fig. 1a, Extended Data Fig. 2f). Double-transgenic embryos were treated with 4-hydroxytamoxifen (4OHT) following two different schedules (Fig. 1b). An ‘early’ group was treated with 4OHT from 8 hpf, a stage before PLM formation, and a ‘late’ group from 30 hpf, a stage just before HSC emergence in the dorsal aorta10,11. These embryos were grown to 3–5 months of age, after which whole kidney marrow cells were analysed by flow cytometry (Fig. 1c). As shown in Fig. 1d, high percentages of switched DsRed+ cells were detected in the ‘early’ group. DsRed− cells were comprised of multiple types of blood lineages (Fig. 1e). In contrast to the ‘early’ schedule, DsRed+ cells were nearly undetectable in the ‘late’ group (Fig. 1d). These results indicate that jam1a is expressed in the shared vascular precursors of HSCs during early somitogenesis stages. The expression of jam1a in HSC precursors was further confirmed by additional lineage-tracing studies using a -5.1jam1a:CreERT2 transgenic animal, which has an extended jam1a promoter/enhancer region (Extended Data Fig. 2g–l).

To examine the function of Jam1a in haematopoiesis, we designed two different morpholino oligonucleotides (MOs), jam1a MOatg (a translation-blocking MO) and MOex7 (a splice-blocking MO) (Extended Data Fig. 3a–e). We first examined the expression of the HSC marker gene runx1 in these morphants. As shown in Fig. 1f, runx1 was detected in the dorsal aorta in uninjected wild-type embryos at 26 hpf. In contrast, runx1 was nearly undetectable in jam1a MOatg- and MOex7-injected embryos at the same stage (Fig. 1g, h). The expression of efnb2a (ephin-B2a, a dorsal aorta marker gene) was unaffected in either morphant (Fig. 1i–k), suggesting that the dorsal aorta is specified normally. To further characterize jam1a morphants, we investigated the expression of additional marker genes. The expression of cmyb (another HSC marker) in the dorsal aorta was largely absent in jam1a morphants (Fig. 1l, m, Extended Data Fig. 3f, g). T-cell colonization of the thymus requires input from HSCs, providing a useful readout for whether HSCs have been specified or not. In jam1a morphants, the expression of rag1 (a marker of immature T cells) was absent in the thymus at 4 days post-fertilization (dpf) (Fig. 1n, o, Extended Data Fig. 3h, i). A truncated isoform of scl (also known as tal1), scl-β, has been shown to mark haemag gon endothelium in the dorsal aorta12. Comparison of scl-α/β and scl-α probes revealed the specific reduction of scl-β in the dorsal aorta in jam1a morphants (Fig. 1p–s). Nascent HSCs can be visualized as cmyb;GFP, kdr;BmpCherry double-positive cells in the ventral floor of the dorsal aorta13. The number of double-positive cells in the dorsal aorta was twelve times lower in jam1a morphants than in wild-type embryos (Extended Data Fig. 4a–c). The expression of gatal (an erythroid marker) and l-plastin (a myeloid marker) at 24 hpf was normal in jam1a morphants, whereas the expression of l-plastin at 4 dpf was reduced in the caudal haematopoietic tissue (CHT) (Extended Data Fig. 4d–f). These results indicate that primitive haematopoiesis is unaffected, but definitive haematopoiesis is defective in jam1a morphants. The vasculature in the trunk was normal in jam1a morphants, whereas development of the vascular plexus in the CHT was slightly abnormal (Extended Data Fig. 4g–j). Development of the pronephros, somite, sclerotome and notochord was unaffected in jam1a morphants (Extended Data Fig. 4k–o). These results indicate that the failure of HSC specification in jam1a morphants is specific and not due to gross...
malformations in adjacent environmental tissues. The effects of MOs are summarized in Supplementary Table 1.

Since jam1a is expressed in PLM cells, we next examined the formation and migration of the PLM in jam1a morphants. The expression of fli1 at 12 hpf was normal in both types of jam1a morphants (Fig. 2a–c), suggesting that PLM formation is unaffected. PLM cells migrate axially and reach the midline by 17 hpf (Fig. 2d). We observed a delay in the migration of PLM cells in both types of jam1a morphants, in that a subset of fli1−cells did not reach the midline by 17 hpf (Fig. 2e, f). We performed time-lapse imaging of PLM cells from 14 hpf using fli1:GFP; phldb1:mCherry double transgenic embryos, where endothelial precursors and somitic cells are labelled by GFP and mCherry expression, respectively. PLM cells in the first wave reached the midline by 15.5 hpf in wild-type embryos, whereas the remaining cells reached the midline by 17.5 hpf to form the ‘vascular cord’ (Fig. 2g, Supplementary Video 1). In jam1a morphants, however, only a few PLM cells reached the midline by 15.5 hpf. Moreover, some PLM cells remained at the lateral borders of the somites at 17.5 hpf, and the vascular cord was discontinuous (Fig. 2h, Supplementary Videos 2 and 3). We examined the morphology of migrating PLM cells. In wild-type embryos, most migrating fli1:GFP+ PLM cells displayed a flattened morphology and appeared to interact closely with the ventral domain of the somite (Fig. 2i). By contrast, PLM cells in jam1a morphants displayed a round shape with relatively little surface contact with the somite (Fig. 2j).

To exclude the possibility of a general developmental delay in jam1a morphants, we enumerated somites at 14 hpf in jam1a MO control- and MOatg-injected embryos. We mainly observed nine somites formed in both jam1a MO control- and jam1a MOatg-injected embryos at this stage, and there was no significant difference in the average numbers of somites between groups (Fig. 2k, l). This indicates that the migration defect observed in jam1a morphants is specific and not due to developmental delay. In zebrafish, Hedgehog (Hh) and Vascular endothelial growth factor a (Vegfa) signalling pathways have been implicated to regulate the migration of PLM cells3,14. In jam1a morphants, however, the expression of shha (sonic hedge-hog a) and vegfa as well as their downstream target efnb2a was unaffected (Fig. 1i–k, Extended Data Fig. 4n–q), indicating that the defect of PLM cell migration in jam1a morphants is independent of the Hh and Vegfa signalling pathways.

Because PLM cells migrate along the ventral domain of the somites, which includes the sclerotomy, it is likely that a binding partner of Jam1a is expressed on the somitic epithelium. Previous studies determined the expression patterns of zebrafish jam genes and their physical binding properties by surface plasmon resonance. These studies showed that Jam1a can bind to Jam2a, Jam2b and Jam3a, but not to Jam1a (homotypically), Jam1b or Jam3b. Moreover, among these 6 jam genes, only jam2a and jam3b are expressed in somites3,15. Therefore, we next investigated whether PLM cells make contact with jam2a+ somitic cells. As shown in Fig. 3a, jam2a was specifically expressed in somites at 16 hpf, a stage when PLM cells are migrating. Quantitative polymerase chain reaction (qPCR) results also showed that jam2a was highly expressed in purified alpha-actin:GFP somitic cells at 14 hpf, whereas jam1a was highly expressed in purified fli1:GFP PLM cells (Fig. 3b). Histological analysis of 16 hpf embryos revealed that migrating fli1:GFP PLM cells were in close contact with jam2a+ somitic cells (Fig. 3c).

To determine if Jam1a can bind to Jam2a, we performed coimmunoprecipitation experiments using transiently transfected Flag-tagged Jam1a (Jam1a–Flag) and haemagglutinin-tagged Jam2a (Jam2a–HA) constructs in HEK293T cells. Anti-Flag immunoprecipitation followed by anti-HA western blotting showed specific binding of Jam1a to Jam2a (Fig. 3d). To further test their interaction, we used a Duolink proximity ligation assay (PLA), which can demonstrate protein–protein interactions in situ by eliciting a fluorescent signal (Fig. 3e). As shown in Fig. 3f, PLA signals were detected in the boundary region between transfected Jam1a–Flag cells and Jam2a–HA cells, revealing the interaction of these proteins in trans. These results suggest that cells of PLM maintain intimate contact with cells of the ventral somite via Jam1a–Jam2a interactions during their migration.

This model predicts that loss of jam2a function would phenocopy the effects in jam1a morphants. We thus examined both HSC specification and PLM cell migration in jam2a MOatg- or MOex5-injected embryos (Extended Data Fig. 5a–d). The expression of runx1 in the dorsal aorta was greatly reduced in both jam2a morphants, whereas efnb2a expression was unaffected (Fig. 3g–j, Extended Data Fig. 5e–l). In addition, axial migration of PLM cells was greatly delayed in both types of jam2a morphants (Fig. 3k, Supplementary Videos 4 and 5), despite only a modest delay in development (Fig. 3l). Migrating fli1:GFP+ PLM cells in jam2a morphants displayed a round shape (Extended Data Fig. 5m), similar to that shown in jam1a morphants (Fig. 2j). The average contact surface area between a PLM cell and the somite was significantly reduced in both jam1a and jam2a morphants compared with uninjected embryos (Extended Data Fig. 5n). The effects of jam2a MOs were further validated in jam2a mutants (jam2aΔ Jun319). Approximately 80% of homozygous jam2aΔ Jun319 embryos showed nearly undetectable expression of runx1 and cmyb in the dorsal aorta and rag1 in the thymus (Extended Data Fig. 6a–g). Moreover, approximately 85% of homozygous jam2aΔ Jun319 embryos showed delayed PLM cell migration compared with wild-type embryos (Extended Data Fig. 6h, i). Formation of the vasculature, however, was grossly normal.
**Figure 2** PLM cell migration is delayed in *jam1a* morphants. **a–f**, The expression of *fli1* in uninjected, *jam1a* MOatg-, or MOex7-injected embryos. Arrowheads indicate a subset of *fli1*+ cells that did not reach the midline by 17 hpf. **g, h**, Time-lapse images of *fli1*:GFP; *phldb1*:mCherry double-transgenic embryos. The regions from the tenth to twelfth somite are shown at each time point. Arrowheads indicate a subset of *fli1*:GFP+ cells that did not reach the midline. **i, j**, Transverse sections of *fli1*:GFP; *phldb1*:mCherry embryos uninjected (15.5 hpf) or injected with *jam1a* MOatg (16 hpf). High magnification views of the boxed regions are shown in the right panels. Dotted lines indicate the contact surface area between PLM cells (arrows) and somitic cells. Bars, 10 μm. **k, l**, The number of somites was counted in *jam1a* MO control- or MOatg-injected embryos at 14 hpf based on the expression of *desma*. The average numbers of somites in embryo groups are shown on each graph. There was no significant difference between *jam1a* MO control- (n = 28) and MOatg-injected embryos (n = 26, P = 0.61, by Student’s t-test). ss, somite-stage. Data are representative of two independent experiments with two different clutches of embryos (a–f, k, l) or three embryos (i, j) or three independent experiments with nine embryos (g, h).

**Figure 3** Loss of somitic *jam2a* phenocopies the *Jam1a* defect. **a**, Expression of *jam2a* at 16 hpf. **b**, Relative expression levels of *jam1a* and *jam2a* in purified *fli1*:GFP+ and *alpha-actin*:GFP+ cells at 14 hpf. Error bars, s.d. **c**, A transverse section of a *fli1*:GFP embryo stained with *jam2a* (purple, white arrowheads) and anti-GFP antibody (brown, black arrowheads) at 16 hpf. The right panel shows a high magnification view of the boxed region. **d**, Co-immunoprecipitation (Co-IP) using anti-Flag antibody. The immunoprecipitates were examined by western blotting using anti-Flag or anti-HA antibody. Inputs represent 10% of cell lysates used in the Co-IP experiment. Arrowheads indicate 40 kDa. **e**, A schematic diagram of the proximity ligation assay (PLA). **f**, A representative result of Duolink PLA. The right panel represents a high magnification view of the boxed region. Arrowheads indicate PLA signal. **g–j**, The expression of *runx1* and *efnb2a* in uninjected or *jam2a* MOatg-injected embryos. Arrowheads indicate the dorsal aorta. **k**, The expression of *fli1* in *jam2a* MOatg-injected embryo at 17 hpf. Arrowheads indicate a subset of *fli1*+ cells that did not reach the midline. **l**, The number of somites was counted in *jam2a* MOatg-injected embryos (n = 26) at 14 hpf based on the expression of *desma*. Average somite number is shown on the graph. ss, somite-stage. Data are representative of two independent experiments with two different clutches of embryos (a–c, g–l) or three independent experiments (d, f).
in jam2a\textsuperscript{hu3319} embryos (Extended Data Fig. 6j–o). These phenotypes are consistent with those in jam1a morphants, suggesting that Jam1a–Jam2a interactions are involved in both PLM cell migration and HSC specification.

Despite a large reduction in embryonic HSC number, approximately 50% of homozygous jam2a\textsuperscript{hu3319} animals were viable and showed almost normal haematopoiesis in the adult kidney (Extended Data Fig. 6p, q). Further studies will be required to understand how haematopoiesis can recover in jam2a\textsuperscript{hu3319} animals during development. Perhaps related to this observation, a dispensable role for Jam2 in adult haematopoiesis has also been reported in mice\textsuperscript{57,18}.

To better understand how both jam1a and jam2a morphants show impaired HSC specification, we considered possible signal transduction mechanisms from the somite. Because our recent work demonstrated that two somitic Notch ligands, Dlc and Dld, are essential for HSC specification\textsuperscript{4}, and because Notch is a juxtacrine signal that requires close contact between adjacent cells, we prioritized analysis of the Notch signalling pathway. To test the hypothesis that Jam1a–Jam2a interactions facilitate Notch signal transmission between the PLM and somite, we first examined the activation of Notch signalling in jam1a morphants using a Notch reporter line, Tp1:GFP, which expresses GFP under the control of tandem Notch responsive elements\textsuperscript{19}. In wild-type embryos, some fl1i:DsRed\textsuperscript{+} endothelial cells strongly expressed Tp1:GFP in the midline at 18 hpf (Fig. 4a). In jam1a morphants, by contrast, most of the fl1i:DsRed\textsuperscript{+} cells showed weak or no expression of the Tp1:GFP reporter at the same stage (Fig. 4b). The expression levels of Tp1:GFP in fl1i:DsRed\textsuperscript{+} cells were further quantified by flow cytometry (Fig. 4c). The mean fluorescence intensity of GFP in the Tp1:GFP\textsuperscript{+} fl1i:DsRed\textsuperscript{+} population was significantly lower in jam1a morphants than in uninjected embryos (Fig. 4d). In wild-type embryos, runx1 is highly expressed in the Tp1:GFP\textsuperscript{+} fl1i:DsRed\textsuperscript{+} population and was significantly downregulated in jam1a morphants (Fig. 4f). At 28 hpf, Tp1:GFP was highly expressed in the dorsal aorta in wild-type embryos (Fig. 4g, h). Interestingly, in jam1a morphants, Tp1:GFP expression was weak and discontinuous along the floor of the dorsal aorta (Fig. 4i, j), the site of HSC emergence\textsuperscript{10,11}. In addition, we observed many apoptotic cells along the aortic floor in jam1a morphants (Fig. 4k, l), suggesting that, in the absence of Notch signalling, HSC precursors fail to be enriched and undergo apoptosis.

To test whether ectopic activation of Notch signalling is sufficient to rescue HSCs in jam1a morphants, we enforced expression of the Notch intracellular domain (NICD), a dominant activator of the Notch pathway\textsuperscript{3}, using combined hsp70:Gal4; UAS:NICD transgenic lines. Heat-shock
induction of NICD at 14 hpf rescued the expression of runx1 in the dorsal aorta in jam1a morphants (Extended Data Fig. 7a, b), similar to that shown previously for rescue of mind bomb (mbi) mutants or wnt16 morphants^{14}. The expression of runx1 was also rescued in jam1a morphants when NICD was induced in the PLM using the 5-1:Gal4 line (Extended Data Fig. 7c, d). Similar results were obtained in jam2a MOatg-injected embryos (Extended Data Fig. 7e, f).

At 15 hpf, migrating 5-1:GFP^{+} cells were observed to make direct contact with dlc^{−} or dld^{−} somitic cells (Extended Data Fig. 8a, b), indicating that PLM cells may receive Notch signalling via presentation of somitic Notch ligands. We observed low activation of Tpl1:GFP in endothelial cells in wnt16 morphants (Extended Data Fig. 8c–g), which show a reduction in somitic dlc and dld^{−}. This suggests that Notch signalling in endothelial cells is activated at least in part by somitic Dlc and/or Dld. We investigated the expression of somitic Notch ligand genes (dlc and dld) as well as aortic Notch receptor and ligand genes (notch1a, notch1b, notch3, dlc and delta-like 4 (dll4)) in jam1a morphants. Importantly, each was expressed normally in jam1a morphants (Fig. 4m–q), suggesting that the defect in Notch signalling in jam1a morphants is due to low Notch signal transmission rather than to misregulation of Notch signalling components. Consistent with this postulate, we observed less contact surface area between migrating PLM cells and the somite in both jam1a and jam2a morphants (Fig. 2i, j, Extended Data Fig. 5m, n), which correlates with low activation of Notch signalling. Our hypothesis is further supported by an additional rescue experiment in which dlc or dld is globally overexpressed in jam1a morphants to present more Notch ligand to HSC precursors. As presented in Fig. 4u, the expression of runx1 in the dorsal aorta was almost fully rescued by co-injection of dld messenger RNA (mRNA) alone with the jam1a MOatg, whereas runx1 expression was only partially rescued following co-injection with dlc mRNA (Fig. 4r–u). Furthermore, the expression of Tpl1:GFP was also restored in the ventral floor of the dorsal aorta by co-injection with dlc or dld mRNA (Extended Data Fig. 9i–p). These data confirm that the impairment of HSC specification in jam1a morphants is caused by inadequate activation of Notch signalling in HSC precursors and suggest that Jam1a and Jam2a normally mediate the physical interaction between these precursors and the somite, which is required for efficient Notch signal transmission (Extended Data Fig. 10).

It has been reported that the overall levels of Notch signal transmission is proportional to adhesion strength between Notch receptor- and ligand-expressing cells^{26}. Our data demonstrate that runx1 is highly expressed in the Tpl1:GFP^{+} population of endothelial cells (Fig. 4e), suggesting that a relatively high level of Notch signalling is required to generate HSC fate. These findings strongly suggest that efficient Notch signal transduction in HSC precursors requires intimate intercellular contact mediated by Jam proteins. Moreover, our data suggest that HSC fate is established much earlier than previously appreciated, during the axial migration of PLM cells, which is well before formation of the dorsal aorta. These new findings may provide key insights into the timing and tissue interactions needed to instruct HSC fate, which should help inform in vitro approaches to generate HSCs from pluripotent stem cells.

METHODS SUMMARY
For morpholino knockdown experiments, zygotes were injected with 1 nl of morpholino oligonucleotides (MOs, GeneTools). MO concentrations used were: jam1a MOatg (100 pmol), jam1a MO control (100 pmol), jam1a MOesc (300 pmol), jam2a MOesc (500 pmol), and wnt16MO2 (5 ng nl^{−1}). Fluorescent images were captured using an SP5 inverted confocal microscope (Leica) as previously described^{27}. Flow cytometry, qPCR, in situ hybridization, histology, Duolink PLA, immunoprecipitation, and western blotting were performed as described in the Methods section.

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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Supplementary Information is available in the online version of the paper.

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Author Contributions I.K., T.S. and D.T. designed research, I.K. generated transgenic lines, performed flow cytometry, cell culture and transfection experiments, analysed data, and wrote the manuscript. I.K. and J.K.-S. performed in situ hybridization and real-time PCR. I.K., J.K.-S. and C.P. generated in situ probes. I.K. and N.F. performed immunoprecipitation and western blotting. I.K. and A.D.K. performed confocal imaging. J.K.-S. performed histological analyses. A.D.K., C.P.-T. and D.T. edited the manuscript.

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Cell preparation and flow cytometry.

Embryos were obtained by macerating the kidney on a stainless steel mesh in 5 ml of ice-cold 2% FBS, cells were cultured in six-well plate with 10% FBS, 200 mM L-glutamine in PBS, and then washed three times with lysis buffer, resuspended in 2× SDS, 0.2 mM DTT, 0.1 M Tris-HCl pH 6.8, 10% glycerol, 2× EDTA, and 0.1 M MgCl2. Cells were then cultured in six-well plate with 10%FBS, 200 mM L-glutamine in Dulbecco’s modified Eagle medium (DME) at 37 °C and 5% CO2.

Immuno precipitation and western blotting. HEK293T cells expressing Jam1a-Flag and/or Jam2a- HA constructs were harvested and lysed in lysis buffer (25 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 150 mM NaCl, 5 mM MgCl2, 20 mM NaOAc, 20% glycerol, 1% Triton X-100, 1 mM dithiothreitol, protease inhibitor). Samples were then centrifuged to remove precipitated proteins and were incubated with anti-Flag M2 agarose antibody (Sigma, A2220) overnight at 4 °C. Samples were washed three times with lysis buffer, resuspended in 2× sample buffer (4% SDS, 0.2 M dithiothreitol, 0.1 M Tris-HCl pH 6.8, 10% glycerol, 20 μg ml−1 bromophenol blue), and boiled for loading. Western blotting was performed as previously described.

Duolink proximity ligation assay. Hela cells expressing Jam1a–Flag and/or Jam2a–HA constructs were co-cultured on a cover slip. Cells were fixed with 4% PFA for 20 min on room temperature, blocked with 1× gelatin-PBS, and then incubated with 1:1,000 mouse anti-HA antibodies (Covance, MMS-101P) or 1:1,000 rabbit anti-Flag antibody (Sigma, F7425) overnight at 4 °C. After washing with 1× PBS, the membrane was incubated with 10,000× duolink conjugated secondary antibody (Jackson ImmunoResearch, 111-035-144) for 45 min on room temperature. After washing with 1× PBS, the membrane was incubated with 1:1,000 goat anti-Flag IgG-HRP conjugated secondary antibody (Jackson ImmunoResearch, 115-035-166) or 1:1,000 goat anti-rabbit IgG-HRP conjugated secondary antibody (Jackson ImmunoResearch, 111-035-144) for 45 min at room temperature. After washing with 1× PBS, the membrane was mounted with Duolink in situ mounting medium with DAPI (Olink, DUO82040). For coloimetric immunochemistry, sections were stained with anti-GFP antibody, followed by staining with 1:1,000 anti-chicken IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, 61-3120) for 1 h at room temperature. Cell preparations were done on paraformaldehyde-fixed tissue sections as previously described.

Embryos were fixed with 4% PFA, embedded in paraffin, and sectioned at 4 μm in thickness. Deparaffinized tissue sections were incubated with blocking solution (1% goat serum, 1% donkey serum, and 0.2% bovine serum albumin in PBS) for 30 min at room temperature and then incubated with 1:1,000 chicken anti-GFP (Aves, GEP-1020). The sections were incubated with 1:1,000 goat anti-RFP antibodies (for DsRed staining, Abcam, ab34771), and/or 1:500 mouse anti-mCherry antibodies (Abcam, ab125096) overnight at 4 °C. For fluorescent immunohistochemistry, sections were incubated with 1:1,000 goat anti-chicken IgG Alexa Fluor 488-conjugated (Molecular Probes, A-11039), 1:1,000 donkey anti-rabbit IgG Alexa Fluor 594-conjugated (Molecular Probes, A-21207), and/or goat anti-mouse IgG Alexa Fluor 594-conjugated (Molecular Probes, A-11055) secondary antibodies for 1 h at room temperature. After washing, sections were mounted with DAPI in situ mounting medium with DAPI (Olink, DUO82040). For coloimetric immunochemistry, sections were stained with anti-GFP antibody, followed by staining with 1:1,000 anti-chicken IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, 61-3120) for 1 h at room temperature. Sections were then developed with 3, 3-diaminobenzidine (DAB) substrate solution (Sigma, D5905) for 3 min at room temperature. After washing, sections were mounted with mounting medium (50% glycerol, 10% gelatin in double-distilled H2O). Whole-mount immunofluorescence was performed as described, using 1:500 mouse anti-Cy3 antibodies (Sigma, M4439) and 1:1,000 donkey anti-mouse IgG Alexa Fluor 488-conjugated (Molecular Probes, A-21202). Haematoxylin and eosin staining was performed on paraffin-embedded tissue sections as previously described.

Time-lapse imaging, embryos were embedded in agarose (1% in E3 medium) containing tritane at a temperature of 28.5 °C. Z-stacks were taken every 196 s. Videos were generated from the z-stacks (ImageJ, Public Domain). Drosophila embryo acridine orange staining was performed as previously described.

Duolink proximity ligation assay. Hela cells expressing Jam1a–Flag and/or Jam2a–HA constructs were co-cultured on a cover slip. Cells were fixed with 4% PFA for 20 min at room temperature, blocked with 1% gelatin-PBS, and then stained with 1:1,000 rabbit anti-Flag and 1:1,000 mouse anti-HA antibodies for 40 min at room temperature. After staining with 1× PBS, the membrane was incubated with 10,000× duolink conjugated secondary antibody (Jackson ImmunoResearch, 115-035-166) or 1:1,000 goat anti-Flag IgG-HRP conjugated secondary antibody (Jackson ImmunoResearch, 111-035-144) for 45 min at room temperature. After washing with 1× PBS, the membrane was mounted with Duolink in situ mounting medium with DAPI.
were displayed as maximum projections. Visible light imaging was performed on a BX-51 microscope using 100× oil objective lens and DP70 digital camera and software (Olympus) or a Leica MZ16 microscope and DFC295 digital camera and software (Leica).

**Morpholino and mRNA injection.** Embryos were injected at the one-cell stage with 1 nl of morpholino oligonucleotides (MOs, GeneTools) and/or mRNA. The MO sequences and concentrations used in this study are as follows: \( \text{jam1a MO}_{\text{atg}}, \text{AGCACACAAAGGCGAAGGTCAACAT} (100 \mu M) \); \( \text{jam1a MO control, AGgAgACAAAcGCcAAGcTCAACAT} (100 \mu M, \text{lowercase letters denote mismatched bases}); \( \text{jam1a MO}_{\text{ex7}}, \text{ATCACCTTTAACAGAGAACAACA} (300 \mu M) \); \( \text{jam2a MO}_{\text{atg}} (300 \mu M) \); \( \text{wnt16 MO}_2 (5 \text{ng/nl}) \). The \( \text{jam2a MO}_{\text{atg}} (300 \mu M) \) and \( \text{wnt16 MO}_2 (5 \text{ng/nl}) \) were used as previously reported. The effects of MOs are summarized in Supplementary Table 1. Capped mRNAs were synthesized from linearized pCS2+ constructs using the mMessage mMachine SP6 kit (Ambion, AM1340), and were injected into embryos at following concentrations: \( \text{dlc}, 50 \text{ ng} \mu \text{l}^{-1} \); \( \text{ddl}, 50 \text{ ng} \mu \text{l}^{-1} \).

**4OHT treatment.** 4-hydroxytamoxifen (4OHT, Sigma, H7904) was dissolved in ethanol as a 25 mM stock solution. Embryos were incubated with 5 \( \mu \text{M} \) of 4OHT in E3 medium. Control embryos were incubated in E3 medium containing 0.02% ethanol. After treatment, the embryos were washed twice in E3 medium and grown as described above.

**Experimental design and statistics.** All experiments comparing treatment groups were made using randomly assigned siblings without investigator blinding. Sample sizes were chosen after estimating effect size, and data were analysed for statistical significance after at least two repeated experiments. All data were analysed by comparison of means using unpaired two-tailed Student’s t-tests. No data were excluded. A value of \( P < 0.01 \) was considered to be statistically significant.
Extended Data Figure 1 | Alignment and phylogenetic analysis of Jam1a.

**a**, The genomic loci of the jam1a and jam1b genes. Arrows indicate the orientation of each gene. **b**, Alignment of zebrafish Jam1a, Jam1b, and human JAM1. The multiple alignment was produced using ClustalW. Asterisks indicate a fully conserved residue. Colons and periods indicate strong and weak similarity, respectively. Dashes indicate gaps. Boxes coloured red, signal peptide (Sp); light blue, immunoglobulin-like domain (Ig); blue, transmembrane domain (TM); green, PDZ-binding domain (PBD); yellow, coiled-coil domain (CCD). Shaded boxes show a cis-dimerization motif. **c**, Phylogenetic analysis of Jam proteins. Cii, *Ciona intestinalis*; Dre, *Danio rerio*; Hsa, *Homo sapiens*; Mmu, *Mus musculus*; Tni, *Tetraodon nigroviridis*; Gga, *Gallus gallus*; Ssc, *Sus scrofa*; Bta, *Bos taurus*; Fca, *Felis silvestris catus*. Cii Jam3 was used as an out-group. The numbers at the relevant branches refer to bootstrap values of 1,000 replications. **d**, Schematic diagrams of human JAM1 and zebrafish Jam1a (left) and Jam1b (right).
Extended Data Figure 2 | The expression of \textit{jam1a} and lineage tracing of \textit{jam1a}-expressing cells. \textbf{a}–\textbf{c}, The expression of \textit{jam1a} at 14, 18 and 24 hpf. Black, blue, and red arrows indicate the posterior lateral mesoderm (PLM), pronephros, and dorsal aorta (DA), respectively. \textbf{d}, Single or two-colour whole-mount \textit{in situ} hybridization with \textit{jam1a} (purple) and/or \textit{fli1} (red) probes. The expression domain of \textit{fli1} merged with that of \textit{jam1a} (arrows). \textbf{e}, qPCR analysis of \textit{jam1a} in purified \textit{fli1:GFP} cells at 14, 17, and 20 hpf. Relative expression levels in each cell population were calculated from the expression levels in the kidney tissue. Error bars, s.d. \textbf{f}, GFP expression of \textit{-2.2jam1a:GFP} at 30 hpf. GFP expression was strongly detected in posterior pronephros, lateral lines, and otic vesicles. \textbf{g}, The expression of CreER\textsuperscript{T2} in \textit{-5.1jam1a:CreER\textsuperscript{T2}} at 24 hpf. \textbf{h}, Three different schedules of 4-hydroxytamoxifen (4OHT) treatment (12–16 hpf, 18–22 hpf, and 24–28 hpf) in \textit{-5.1jam1a:CreER\textsuperscript{T2}, bactin2loxP-BFP-loxP-DsRed}. The numbers indicate hour post fertilization (hpf), and red insets in the blue arrows indicate the period of the 4OHT treatment. \textbf{i}, Flow cytometric analysis of kidney marrow cells in 12–16 hpf group (left, \textit{n} = 5), 18–22 hpf group (middle, \textit{n} = 5), and 24–28 hpf group (right, \textit{n} = 2) at 2 months of age. One 12–16 hpf animal showed switched DsRed\textsuperscript{+} cells in kidney marrow cells. \textbf{j}, DsRed\textsuperscript{+} cells from 12–16 hpf kidneys are distributed in all blood cell populations, including neutrophils and eosinophils (N+E), precursors and monocytes (P+M), and lymphocytes and thrombocytes (L+T). \textbf{k}, \textbf{l}, Confocal imaging of the DA and caudal haematopoietic tissue (CHT) in a 12–16 hpf embryo at 48 hpf. Right panels show high magnification views of the boxed regions in left panels. Embryos are oriented with anterior to the left. There are many ‘switched’ DsRed\textsuperscript{+} cells in the ventral floor of the DA and in the CHT (arrowheads). Bars, 20 \textmu m. Data are representative of two independent experiments with two different clutches of embryos (\textbf{a}–\textbf{e}, \textbf{g}) or eight embryos (\textbf{k}, \textbf{l}) or pooled from two independent experiments (\textbf{i}, \textbf{j}).
Extended Data Figure 3 | Characterization of \textit{jam1a} MOex7-injected embryos. a, RT–PCR results from \textit{jam1a} MOex7-injected embryos. cDNA from embryos un.injected or injected with various doses of \textit{jam1a} MOex7 (100–500 μM) was subjected to RT–PCR analysis using specific primers, which amplify from exon 4 to 10 of \textit{jam1a}. \textit{ef1a} was used as a control. The expected size of PCR products in uninjected embryos is 746 base pairs (bp) (black arrow). Exon 7 (108 bp)-skipped products were detected in \textit{jam1a} MOex7-injected embryos (red arrow). The dose of 300 μM was used in this study. b, Exon 7-skipped products were verified by sequencing. The dotted line indicates the junctions between exon 6 and exon 7 (uninjected, upper panel) or exon 8 (\textit{jam1a} MOex7, lower panel). c, A schematic diagram of the mRNA splicing in \textit{jam1a} MOex7-injected embryos. The red bar indicates the binding site of \textit{jam1a} MOex7. d, Schematic diagrams of Jam1a protein in wild-type (left) or \textit{jam1a} MOex7-injected embryos (right). Since exon 7 encodes the transmembrane domain (TM), \textit{jam1a} MOex7-injected embryos express a mutant protein lacking the TM. Sp, signal peptide; Ig, immunoglobulin-like domain; PBD, PDZ-binding domain. e, The relative expression of wild-type \textit{jam1a} mRNA in uninjected or \textit{jam1a} MOex7 (300 μM)-injected embryos at 24 hpf. The reverse primer was designed in exon 7. Error bars, s.d. f–i, The expression of \textit{cmyb} and \textit{rag1} in uninjected or \textit{jam1a} MOex7-injected embryos. Arrowheads indicate the dorsal aorta (f, g) or the thymus (h, i). Data are representative of two independent experiments with two different clutches of embryos (a, e–i).
Extended Data Figure 4 | jam1a is required for HSC specification.

a–c. Fluorescently labelled HSCs in cmyb:GFP, kdrl:mCherry. The number of cmyb:GFP, kdrl:mCherry double-positive cells in the dorsal aorta (DA) were counted in uninjected or jam1a MOatg-injected embryos at 48 hpf (arrows). Embryos are oriented with anterior to the left. The average number of double-positive cells is significantly lower in jam1a MOatg-injected embryos (n=10) compared with uninjected embryos (n=10), *P<0.001 by Student’s t-test; error bars, s.d. d–q. The expression of gata1 (an erythroid marker), l-plastin (a myeloid marker) at 24 hpf or 4 dpf, kdrl (a pan-endothelial marker) in the trunk or caudal haematopoietic tissue (CHT), tbx20 (a marker for the roof of DA), flt4 (a vein marker), cdh17 (a pronephros marker), desma (a somite marker), nkx3.1 (a sclerotome marker), shha (a notochord marker) at 16 hpf or 26 hpf, and vegfa at 17 hpf or 26 hpf in uninjected or jam1a MOatg-injected embryos. Arrowheads indicate CHT (f), DA (i), or notochord (o). Data are representative of two independent experiments with ten embryos (a–c) or two different clutches of embryos (d–q).
Extended Data Figure 5 | Characterization of jam2a MOex5-injected embryos. a, RT–PCR results from jam2a MOex5-injected embryos. cDNA from embryos uninjected or injected with various doses of jam2a MOex5 (200–600 μM) was subjected to RT–PCR analysis using specific primers, which amplify from exon 4 to 10 of jam2a. ef1a was used as a control. The expected size of PCR products in uninjected embryos is 537 base pairs (bp) (black arrow). Intron 4 (72 bp)-trapped products were detected in jam2a MOex5-injected embryos (red arrow). The dose of 400 μM was used in this study. b, Intron 4-trapped products were verified by sequencing. The dotted line indicates the junctions between exon 4 and exon 5 (uninjected, upper panel) or intron 4 (jam2a MOex5, lower panel). c, A schematic diagram of the mRNA splicing in jam2a MOex5-injected embryos. The red bar indicates the binding site of jam2a MOex5. d, Schematic diagrams of Jam2a protein in wild-type (left) or jam2a MOex5-injected embryos (right). Since intron 4 contains an in-frame stop codon, jam2a MOex5-injected embryos express a truncated mutant protein. Sp, signal peptide; Ig, immunoglobulin-like domain; TM, transmembrane domain; PBD, PDZ-binding domain. e–l, The expression of runx1, efnb2a, cmyb, and ragl in uninjected or jam2a MOex5-injected embryos. Arrowheads indicate the dorsal aorta (e–j) or the thymus (k, l). m, A transverse section of a fltl:GFP; phldb1:mCherry embryo injected with jam2a MOex5 at 16 hpf. A high magnification view of the boxed region is shown in the right panel. A dotted line in the right panel indicates the contact surface area between fltl:GFP+ PLM cells and phldb1:mCherry+ somitic cells. Bars, 10 μm. n, The average contact surface area per cell (μm² per cell) was calculated from at least one hundred fltl:GFP+ cells in an embryo, and the averages were obtained from three embryos of each. *P < 0.01, by Student’s t-test. Error bars, s.d. Data are representative of two independent experiments with two different clutches of embryos (a, e–l) or three embryos (m, n).
Extended Data Figure 6 | jam2a is required for HSC specification. a, A schematic diagram of the preparation of jam2a mutant (jam2ahu3319) embryos. Embryos from an incross of genotyped homozygous (homo) jam2ahu3319 animals were examined by whole-mount in situ hybridization (WISH). b–g, The expression of runx1 and cmyb in the dorsal aorta (DA) and rag1 in the thymus in wild-type or jam2ahu3319 embryos. The numbers shown in each panel indicate the frequency of embryos showing each expression pattern. h, i, The expression of fli1 at 18 hpf in wild-type or jam2ahu3319 embryos. Arrows indicate a subset of fli1+ cells that did not reach the midline. j–o, The expression of endothelial marker genes (kdrl, efnb2a, and flt4) in wild-type or jam2ahu3319 embryos at 28 hpf. Approximately 90% of embryos showed normal vascular plexus, while the rest of embryos showed a reduction of efnb2a and kdrl. Arrowheads indicate the DA (b–e, l, m), the thymus (f, g), or posterior cardinal vein (n, o). p, Histological analysis of the adult kidney in a wild-type or jam2ahu3319 animal at 2 months of age. Many blood cells are observed in the marrow area. Haematoxylin and eosin (HE) staining. q, Representative results of flow cytometric analysis of kidney marrow cells from a wild-type or jam2ahu3319 animal at 3 months of age. All blood cell populations are detected in jam2ahu3319 animals. L+T, lymphocytes and thrombocytes; N+E, neutrophils and eosinophils; P+M, precursors and monocytes. Data are representative of two independent experiments with two different clutches of embryos (b–o) or three different animals (p, q).
Extended Data Figure 7 | Enforced expression of the Notch intracellular domain rescues HSCs in jam1a or jam2a morphants. Heat-shock (HS) (hsp70:Gal4, a, b, e) or endothelial (fli1:Gal4, c, d, f) induction of Notch intracellular domain (NICD) in uninjected, jam1a MOatg-, or jam2a MOatg-injected embryos. Left panels show whole-mount immunofluorescence visualization of Myc-tagged NICD, and right panels show the expression of runx1 at 26 hpf. Arrowheads indicate the dorsal aorta. Data are representative of two independent experiments with two different clutches of embryos (a–f).
Extended Data Figure 8 | Somitic Dlc and Dld are involved in the activation of endothelial Notch signalling. a, b, Transverse sections of fli1:GFP embryos stained with dlc or dld (purple) and anti-GFP antibody (brown) at 15 hpf. Right panels show high magnification views of the boxed regions. Migrating fli1:GFP cells (black arrowheads) are in contact with dlc or dld somitic cells (white arrowheads). c–e, Flow cytometric analysis of Tp1:GFP; fli1:DsRed embryos uninjected or injected with wnt16 MO at 22 hpf. Representative results of flow cytometric analysis (c), the mean fluorescent intensities of GFP in Tp1:GFP; fli1:DsRed populations (d), and the percentages of Tp1:GFPhigh in fli1:DsRed populations (e) are shown. Blue gates and red circles indicate the Tp1:GFP−; fli1:DsRed− and Tp1:GFPhigh; fli1:DsRed− population, respectively. *P < 0.01, by Student’s t-test. Error bars, s.d. f, g, Lateral views of the dorsal aorta (DA) in Tp1:GFP; fli1:DsRed embryos uninjected or injected with wnt16 MO at 28 hpf. Arrows indicate the low activation of Tp1:GFP in the ventral floor of the DA. Data are representative of two independent experiments with four embryos (a, b), eight embryos (f, g), or four different clutches of embryos (c–e).
Extended Data Figure 9 | Aortic Tp1:GFP expression is restored by overexpression of dlc or dld in jam1a morphants. a–h. The aortic expression of notch1b, notch3, dlc, and dll4 in uninjected or jam1a MOatg-injected embryos at 26 hpf. Arrowheads indicate the dorsal aorta (DA). i–p. Lateral views of the DA in Tp1:GFP (i–l) and transverse sections of Tp1:GFP; fli1:DsRed (m–p) at 28 hpf. Embryos were uninjected, injected with jam1a MOatg alone, or co-injected with jam1a MOatg and dlc or dld mRNA. Arrows indicate relatively low activation of Tp1:GFP in the ventral floor of the DA. The expression of Tp1:GFP was restored in the ventral floor of the DA by co-injection with dlc or dld. Bars, 10 μm. Data are representative of two independent experiments with two different clutches of embryos (a–h), eight embryos (i–l), or three embryos (m–p).
Extended Data Figure 10 | A model of Notch signal transduction in HSC precursors. Jam1a+ PLM cells initially flank the somites then migrate to the midline along the ventral face of the somite, where Jam2a and the Notch ligands Dlc and Dld are expressed. Binding of Jam1a and Jam2a in trans is required for transmission of Notch signals into the PLM derivatives that subsequently generate aortic haemogenic endothelium (left side). In jam1a-deficient embryos, although PLM cells arise and initially migrate normally, their migration is delayed upon contact with the somite. Moreover, they show low activation of Notch signalling due to poor interaction with the somite, resulting in the failure of HSC specification in the aortic floor (right side).