Figures and figure supplements

Hand2 inhibits kidney specification while promoting vein formation within the posterior mesoderm

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Figure 1. hand2 inhibits pronephron formation. (A–P) Dorsal views, anterior to the left, of pronephron schematics (A, E, I, M), wild-type embryos (B, F, J, N), han<sup>66</sup> mutant embryos (C, G, K, O), and hand2-overexpressing embryos (D, injected with hand2 mRNA; H, L, P, carrying Tg<hsp70-hand2-2A-mcherry>, abbreviated hs:hand2) at 24 hpf. In schematics (A, E, I, M), colored regions correspond to area of pronephron gene expression. In situ hybridization demonstrates that <i>atp1a1a</i>.4 (A–D) and <i>cdh17</i> (E–H) are expressed throughout the pronephron tubules, <i>slc12a3</i> (I–L) is expressed in the distal late segments of the pronephron tubules, and <i>lhx1a</i> (M–P) is expressed in the glomerular precursors (arrows, N), as well as overlying spinal neurons (asterisks, N). Compared to wild-type (B, F, J, N), gene expression is expanded in han<sup>66</sup> mutants (C, G, K, O) and reduced in hand2-overexpressing embryos (D, H, L, P). Of note, injection of a hand2 translation-blocking morpholino caused effects on pronephron formation similar to those seen in han<sup>66</sup> mutants (data not shown). Scale bars represent 100 μm. (Q, R) Transverse sections through wild-type (Q) and han<sup>66</sup> mutant (R) pronephron tubules at 24 hpf. Dashed lines outline the tubule and asterisks indicate individual tubule cells. (S, T) Bar graphs indicate the average number of tubule cells per cross-section (S) and the average tubule area per cross-section (T) in wild-type and han<sup>66</sup> mutant embryos; error bars indicate standard deviation. Asterisks indicate statistically significant differences compared to wild-type (p<0.0001, Student’s t test; n = 18). DOI: 10.7554/eLife.19941.003

The following source data is available for figure 1:

**Source data 1.** Number of tubule cells per cross-section.
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**Source data 2.** Area of pronephron tubule in cross-section.
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Figure 2. *hand2* inhibits IM production. (A–C) Dorsal views, anterior to the left, of the posterior mesoderm at the 11 somite stage. In situ hybridization depicts normal expression of *pax2a* in the IM (arrows) of wild-type embryos (A), widened expression in *han<sup>66</sup>* mutants (B), and lack of expression in *hand2*-overexpressing embryos (hs:*hand2*) (C). Expression in the spinal neurons (asterisk) is unaffected by altered *hand2* function. Scale bar represents 100 μm. (D–F) Pax2a immunofluorescence in the posterior mesoderm of wild-type (D), *han<sup>66</sup>* mutant (E), and hs:*hand2* (F) embryos at the 12 somite stage. Dorsal views, anterior to the left, are three-dimensional reconstructions of flat-mounted embryos from which the yolk and anterior tissues have been dissected away. Scale bar represents 100 μm. (D’–F’) Magnification of 250 μm long regions from (D–F) used for quantification of the number of Pax2a<sup>+</sup> cells in wild-type (D’), *han<sup>66</sup>* mutant (E’), and hs:*hand2* (F’) embryos. White dots indicate Pax2a<sup>+</sup> nuclei. Intensity of staining varied from strong (for example, green arrows) to weak (for example, yellow arrows). Scale bar represents 25 μm. (G) Bar graph indicates the average number of Pax2a<sup>+</sup> cells per 100 μm of IM in wild-type, *han<sup>66</sup>* and hs:*hand2* mutant embryos; error bars indicate standard deviation. Asterisks indicate a statistically significant difference compared to wild-type (p<0.0001, Student’s t test; n=13 for wild-type, n=10 for *han<sup>66</sup>*, and n=19 for hs:*hand2*).

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The following source data is available for figure 2:

**Source data 1.** Pax2a<sup>+</sup> cells in wild-type, *han<sup>66</sup>* and hs:*hand2* intermediate mesoderm.

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Figure 2—figure supplement 1. hand2 inhibits IM production. (A–C) Dorsal views, anterior to the top, of the posterior mesoderm at the 10 somite stage. In situ hybridization depicts normal expression of \textit{lhx1a} in the IM (arrows) of wild-type embryos (A), widened expression in \textit{han}\textsuperscript{s6} mutants (B), and reduced expression in \textit{hand2}-overexpressing embryos (C). Expression in the notochord (asterisk) is unaffected by altered \textit{hand2} function. Scale bar represents 100 \textmu m.

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Figure 2—figure supplement 2. Comparable proliferation in the IM of wild-type and han\textsuperscript{s6} mutant embryos. (A, B) Immunofluorescence for Pax2a and phospho-Histone H3 (pH3) in wild-type (A) and han\textsuperscript{s6} mutant (B) embryos at the 6 somite stage; dorsal views, anterior to the left, are three-dimensional reconstructions of 400 \textmu m long regions used for quantification of the numbers of Pax2a\textsuperscript{+} (A', B') and pH3\textsuperscript{+} (A'', B'') cells. Arrows indicate Pax2a\textsuperscript{+} pH3\textsuperscript{+} cells. Scale bar represents 50 \textmu m. (C) Table compares the number of Pax2a\textsuperscript{+} pH3\textsuperscript{+} cells per 100 \textmu m of IM, the number of Pax2a\textsuperscript{+} cells per 100 \textmu m of IM, and the Proliferation Index (Pax2a\textsuperscript{+} pH3\textsuperscript{+} / Pax2a\textsuperscript{+} x 100).
Figure 2—figure supplement 2 continued

and the proliferation index (Pax2a⁺ pH3⁺ cells / Pax2a⁺ cells X 100) in wild-type and han⁺⁄⁻ mutant embryos. Data shown represent the average and standard deviation for each value. While there was a statistically significant increase in the number of Pax2a⁺ cells in han⁺⁄⁻ mutants compared to wild-type (p<0.0001), no significant differences were observed for the number of Pax2a⁺ pH3⁺ cells (p=0.1461) or the proliferation index (p=0.0654).

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The following source data is available for figure 2:

Figure supplement 2—Source data 1. Pax2a⁺ pH3⁺ cells in wild-type and han⁺⁄⁻ intermediate mesoderm.
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Figure 3. Pronephron development is susceptible to hand2 overexpression prior to the 10 somite stage. (A–E) Dorsal views, anterior to the left, of in situ hybridization for \textit{atp1a1a.4} at 24 hpf depict a range of severity of pronephron defects, ranging from absence of the pronephron (A) to unaffected (E). Tg(hsp70:FLAG-hand2-2A-mCherry) embryos were subjected to heat shock at the tailbud, 2 somite, 6 somite, or 10 somite stages, and the consequences on pronephron development were scored at 24 hpf. Percentages indicate the distribution of phenotypes produced by each treatment; the number of embryos examined is in the right-hand column. Heat shock at later stages resulted in more mild loss of \textit{atp1a1a.4} expression in the tubule, and heat shock at the 10 somite stage did not disrupt \textit{atp1a1a.4} expression in the tubule. Scale bar represents 100 μm.

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Figure 4. *hand2* expression in the posterior lateral mesoderm. (A–D, F–I) Two-color fluorescent in situ hybridization (A, C, F, H) and immunofluorescence (B, D, G, I) label components of the posterior mesoderm in dorsal views, anterior to the left, of three-dimensional reconstructions, as in Figure 2D–E. In embryos containing transgenes in which GFP expression is driven by the regulatory elements of *hand2* (B, G) or *etv2* (D, I), anti-GFP immunofluorescence was used to enhance visualization. Tg(etv2:egfp) expression was also observed in the midline neural tube, as previously reported (Proulx et al., 2010). Scale bar represents 100 μm. (E, J) Schematics depict posterior mesoderm territories, dorsal views, anterior to the left; *hand2*-expressing cells, IM, and medial vessel/blood progenitors are shown at 2–10 somites (E) and at 11–13 somites (J), together with lateral vessel progenitors. (A–D) *hand2* is expressed lateral to the IM at the 2–10 somite stages. Embryos shown are at the 10 somite stage; similar expression patterns were seen at earlier stages. (A, B) *hand2* is expressed lateral to the IM markers *lhx1a* (A) and Pax2a (B). (C) *hand2* is expressed lateral to *tal1*, a marker of blood and vessel progenitors; note the unlabeled gap between expression territories. (D) A marker of vessel progenitors, Tg(etv2:egfp), lies medially adjacent to Pax2a. (F–I) Vessel progenitors arise at the interface between *hand2*-expressing cells and the IM at the 11–13 somite stages. (F, G) *hand2* is expressed lateral to the IM marker Pax2a. (H) *hand2* is expressed lateral to a second territory of *tal1* expression; note the presence of lateral *tal1*-expressing cells (arrows) immediately adjacent to *hand2* expression. (I) The IM lies between two territories of *etv2* expression; the more lateral *etv2*-expressing cells (arrows) are lateral to the IM.

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Figure 5. hand2 promotes vessel progenitor development. (A–C, F–H, K–M) In situ hybridization depicts etv2 (A–C), tal1 (F–H) and gata1 (K–M) expression in wild-type (A, F, K), han$^{66}$ mutant (B, G, L) and hand2-overexpressing (C, H, M) embryos; dorsal views, anterior to the left, at the 12 somite stage. (A, F) etv2 and tal1 are expressed in relatively medial and lateral (arrows) territories on each side of the wild-type embryo. In han$^{66}$ embryos (B, Figure 5 continued on next page
G), only the medial territory is present. In hand2-overexpressing embryos (C, H), expression of both etv2 and tal1 is increased, but it is not possible to distinguish whether this represents an increase in the medial or the lateral vessel progenitor populations, since no markers exist that distinguish these two groups of progenitors. (K–M) gata1 expression is equivalent in wild-type (K) and han^sh (L) embryos, but it is decreased in hand2-overexpressing embryos (M). Scale bar represents 100 μm. (D, E, I, J) Fluorescent in situ hybridization depicts the relationship of etv2 and pax2a expression in wild-type (D, D') and hand2 morphant (hand2 MO) embryos (E, E'), and the relationship of tal1 and hand2 expression in wild-type (I, I') and hand2 MO (J, J') embryos; dorsal views, anterior to the left, at the 12 somite stage. Medial and lateral (arrows) territories of etv2 expression flank pax2a in wild-type embryos (D, D'). The lateral territory of expression is absent in hand2 morphants (E, E'). The lateral territory (arrows) of tal1 expression is located at the medial border of hand2 expression in wild-type embryos (I, I'), but is absent in hand2 morphants (J, J'). Note that we observed variable thickness of tal1 expression in its medial territory of expression.

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Figure 5—figure supplement 1. Presence of medial vessel and blood progenitors is unaffected in han<sup>66</sup> mutants. (A–F) In situ hybridization depicts expression of etv2 (A, B), tal1 (C, D), and gata1 (E, F) in wild-type (A, C, E) and han<sup>66</sup> mutant (B, D, F) embryos; dorsal views, anterior to the top, of the posterior mesoderm at the 6 somite stage. Expression of all three markers appears unperturbed by loss of hand2 function at this stage. Scale bar represents 100 μm.
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Figure 6. hand2 promotes proper vein formation. (A–I) In situ hybridization depicts expression of mrc1a (A–C), flt4 (D–F) and efnb2a (G–I) in wild-type (A, D, G), han<sup>86</sup> mutant (B, E, H) and hand2-overexpressing (C, F, I) embryos; lateral views, anterior to the left, at 24 hpf (A–C, G–I) and the 20 somite stage (D–F).

Figure 6 continued on next page

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stage (D–F). (A–C) mrc1a expression in the posterior cardinal vein (blue arrow) was present in wild-type (A), absent in han^{hs} mutant (B), and increased in hand2-overexpressing (C) embryos. Expression in the posterior blood island (red arrowhead) was grossly unaffected. (D–F) flt4 expression in the posterior cardinal vein (blue arrow) was present in wild-type (D), absent in han^{hs} mutant (E), and increased in hand2-overexpressing (F) embryos. Expression in the dorsal aorta (red arrow) was grossly unaffected. (G–I) efnb2a expression in the dorsal aorta (red arrow) was present in wild-type (G), grossly unaffected in han^{hs} mutant (H), and slightly increased in hand2-overexpressing (I) embryos. (J–O) Lateral views of three-dimensional reconstructions of Tg(flk1:ras-mcherry) expression in the vasculature of wild-type (J, M) and han^{hs} mutant (K, N) embryos at 28 hpf (J, K) and 32 hpf (M, N). Pictured region in (J, K) is the area of the trunk boxed in the schematic (L); pictured region in (M, N) is the area of the tail boxed in the schematic (O). Both posterior cardinal vein (blue arrow) and dorsal aorta (red arrow) were present in wild-type (J) and han^{hs} mutant (K) embryos. In contrast to wild-type (M), the caudal venous plexus (arrowhead) fails to undergo proper remodeling in han^{hs} mutants (N). Scale bars represent 100 μm.
Figure 7. Increased presence of Pax2a in hand2-expressing cells of hand2 mutants. (A–B) In situ hybridization depicts presence of hand2 expression in hand2 morphants (B); dorsal views, anterior to the left, at the 10 somite stage. There is no evident loss of hand2-expressing cells in hand2 morphants; Figure 7 continued on next page.
moreover, hand2 expression levels appear higher in the context of hand2 loss-of-function. Scale bar represents 100 μm. (C–D) Immunofluorescence for Pax2a and GFP in wild-type (C) and han6 mutant (D) embryos, both carrying Tg(hand2:EGFP), dorsal views, anterior to the left, of three-dimensional reconstructions at the 12 somite stage, as in Figure 4G. (C’–D’’) Magnification of 250 μm long regions from (C) and (D) used for quantification of the numbers of GFP+ and Pax2a+ cells in wild-type and han6 mutant embryos. Yellow dots indicate GFP+ cells, white dots indicate Pax2a+ nuclei, and examples of GFP+ Pax2a+ cells are indicated by white arrows. Intensity of Pax2a+ staining varied from strong (for example, green arrows) to weak (for example, yellow arrows). Scale bars represent 100 μm (C, D) and 25 μm (C’–D’’). (E) Bar graph indicates the average numbers of GFP+ cells and GFP+ Pax2a+ cells per 100 μm of IM in wild-type and han6 mutant embryos; error bars indicate standard deviation. Asterisk indicates a statistically significant difference compared to wild-type (p<0.0001, Student’s t test; n = 13 for wild-type and n = 10 for han6).

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The following source data is available for figure 7:

Source data 1. GFP+ and Pax2a+ GFP+ cells in wild-type and han6 intermediate mesoderm.
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Figure 7—figure supplement 1. Increased hand2:gfp expression in han^{ls}. Immunofluorescence for GFP in wild-type (left) and han^{ls} mutant (right) embryos, both carrying Tg(hand2:EGFP), dorsolateral views, anterior to the top, at 12 somite stage. Levels of hand2:gfp expression appear higher in the context of hand2 loss-of-function.

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Figure 8. hand2 and osr1 act in opposing, parallel pathways to regulate pronephron development. (A–A'') Fluorescent in situ hybridization depicts overlap (A) of hand2 (A') and osr1 (A'') expression in wild-type embryos; dorsal views, anterior to the left, of three-dimensional reconstructions at the 6 somite stage. (B–I) In situ hybridization depicts pax2a (B–E) and atp1a1a.4 (F–I) expression at 24 hpf in wild-type embryos (B, F), han66 mutant embryos (C, G), osr1 morphant (osr1 MO) embryos (D, H) and han66 mutant embryos injected with osr1 morpholino (han66 + osr1 MO) (E, I); dorsal views, anterior to the left. (B–E) Compared to wild-type (B), pax2a expression in the glomerular and neck precursors (arrow) was expanded in 100% of han66 mutants (C, n=11), absent (48%) or reduced (48%) in osr1 morphants (D, n=25), and relatively normal in han66 + osr1 MO embryos (E, n=11). While the extent of marker expression was generally comparable to wild-type in the double loss-of-function embryos, the stereotypic patterning of this population was often somewhat disrupted. Expression in overlying spinal neurons (asterisk) was unaffected. (F–I) Compared to wild-type (F), atp1a1a.4 expression in the pronephric tubules was wide in 85% of han66 mutants (G, n=13), while many osr1 morphants (H, n=133) had tubules with shortened anterior expression (18%) or tubules with segmental losses (35%), and 47% of osr1 morphants had a wild-type appearance. 85% of han66 + osr1 MO embryos (I, n=46) resembled wild-type, whereas 11% had a shortened anterior tubule and 4% had segmental losses in the tubules. Scale bars represent 100 μm.

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Figure 8—figure supplement 1. Expression patterns of osr1 and hand2 appear unaffected by loss of each other’s function. (A–D) In situ hybridization depicts expression of osr1 (A, B) and hand2 (C, D) in the posterior mesoderm of wild-type (A, C), han^{s6} mutant (B), and osr1 MO (D) embryos, dorsal views, anterior to the top, of the posterior portion of the embryo at the 4 somite (A, B) and 6 somite (C, D) stages. (A, B) Loss of hand2 function does not appear to affect osr1 expression. (C, D) Loss of osr1 function does not appear to affect hand2 expression. Scale bar represents 100 μm.

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