

1 **Hand2 Inhibits Kidney Specification While Promoting Vein Formation Within the**  
2 **Posterior Mesoderm**

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17

1 **ABSTRACT**

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3 Proper organogenesis depends upon defining the precise dimensions of organ  
4 progenitor territories. Kidney progenitors originate within the intermediate mesoderm  
5 (IM), but the pathways that set the boundaries of the IM are poorly understood. Here, we  
6 show that the bHLH transcription factor Hand2 limits the size of the embryonic kidney  
7 by restricting IM dimensions. The IM is expanded in zebrafish *hand2* mutants and is  
8 diminished when *hand2* is overexpressed. Within the posterior mesoderm, *hand2* is  
9 expressed laterally adjacent to the IM. Venous progenitors arise between these two  
10 territories, and *hand2* promotes venous development while inhibiting IM formation at this  
11 interface. Furthermore, *hand2* and the co-expressed zinc-finger transcription factor *osr1*  
12 have functionally antagonistic influences on kidney development. Together, our data  
13 suggest that *hand2* functions in opposition to *osr1* to balance the formation of kidney and  
14 vein progenitors by regulating cell fate decisions at the lateral boundary of the IM.

15

1 **IMPACT STATEMENT**

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3           The Hand2 transcription factor regulates the dimensions of the kidney by

4 controlling cell fate decisions at the interface between organ fields.

5

## 1 INTRODUCTION

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3           Organs arise from precisely defined territories containing progenitor cells with  
4 specific developmental potential. Distinct progenitor territories often abut one another,  
5 and communication at the interfaces between neighboring territories acts to refine their  
6 boundaries (Dahmann et al., 2011). This process delineates the final dimensions of each  
7 territory and, subsequently, influences the sizes of the derived organs. Boundary  
8 refinement is generally thought to be mediated by interplay between opposing inductive  
9 and suppressive factors (Briscoe and Small, 2015). In many cases, however, the  
10 identification of and interactions among these factors remain elusive.

11           Kidney progenitor cells originate from the intermediate mesoderm (IM), a pair of  
12 narrow bilateral stripes within the posterior mesoderm, flanked by the lateral plate  
13 mesoderm (LPM) that gives rise to vessels and by the paraxial mesoderm that gives rise  
14 to bone, cartilage, and skeletal muscle. The mechanisms that determine the dimensions  
15 of the stripes of IM are not fully understood. Several conserved transcription factors are  
16 expressed in the IM and are required for its development, including *Lhx1/Lim1*, *Pax2*,  
17 and *Osr1/Odd1* (Dressler and Douglass, 1992; James et al., 2006; Krauss et al., 1991;  
18 Toyama and Dawid, 1997; Tsang et al., 2000; Wang et al., 2005). Studies in chick have  
19 indicated essential roles for the LPM, paraxial mesoderm, and surface ectoderm in  
20 regulating the expression of these transcription factors (James and Schultheiss, 2003;  
21 Mauch et al., 2000; Obara-Ishihara et al., 1999). Furthermore, TGF-beta signaling acts in  
22 a dose-dependent manner to pattern the medial-lateral axis of the posterior mesoderm: for  
23 example, low levels of BMP signaling promote IM formation while high levels of BMP

1 signaling promote LPM formation (Fleming et al., 2013; James and Schultheiss, 2005).  
2 Beyond these insights, the pathways that set the boundaries of the IM and distinguish this  
3 territory from its neighbors are largely unknown.

4         Several lines of evidence have suggested that a carefully regulated refinement  
5 process is required to sharpen the boundary between the IM and LPM. In chick, mouse,  
6 and *Xenopus*, *Osr1* and *Lim1* are expressed in both the LPM and IM before becoming  
7 restricted to the IM, implying the existence of a mechanism that acts to exclude IM gene  
8 expression from the LPM territory (Carroll and Vize, 1999; James et al., 2006; Mugford  
9 et al., 2008; Tsang et al., 2000). Additional data have hinted at an antagonistic  
10 relationship between the IM and LPM lineages (Gering et al., 2003; Gupta et al., 2006):  
11 for example, overexpression of vascular and hematopoietic transcription factors (*tall* and  
12 *lmo2*) induces ectopic vessel and blood specification while inhibiting IM formation  
13 (Gering et al., 2003). Along the same lines, zebrafish *osr1* morphants exhibit disrupted  
14 pronephron formation together with expanded venous structures (Mudumana et al.,  
15 2008). Despite these indications of interconnected IM and LPM development, the  
16 network of factors that link these processes has not been fully elucidated.

17         Here, we establish previously unappreciated roles for the bHLH transcription  
18 factor Hand2 in both IM and vessel formation. Prior studies of Hand2 have focused on  
19 its functions in other tissues, including the heart, limb, and branchial arches (e.g. (Charité  
20 et al., 2000; Fernandez-Teran et al., 2000; Funato et al., 2009; Miller et al., 2003;  
21 Srivastava et al., 1997; Yanagisawa et al., 2003; Yelon et al., 2000). Although Hand2 is  
22 also expressed in the posterior mesoderm (Angelo et al., 2000; Fernandez-Teran et al.,  
23 2000; Srivastava et al., 1997; Thomas et al., 1998; Yelon et al., 2000; Yin et al., 2010), its

1 influence on patterning this tissue has not been extensively explored. Through both loss-  
2 of-function and gain-of-function studies, we find that *hand2* limits the size of the kidney  
3 by repressing IM formation while promoting venous progenitor formation. *hand2* is  
4 expressed laterally adjacent to the IM, and a set of venous progenitors arise at the  
5 interface between the *hand2*-expressing cells and the IM. Ectopic expression of IM  
6 markers within the *hand2*-expressing territory in *hand2* mutants suggests that *hand2*  
7 establishes the lateral boundary of the IM through direct inhibition of IM fate acquisition.  
8 Finally, genetic analysis indicates that *hand2* functions in opposition to *osr1* to control  
9 kidney dimensions. Together, our data demonstrate a novel mechanism for defining  
10 territory boundaries within the posterior mesoderm: *hand2* represses IM formation to  
11 establish its lateral boundary while promoting venous progenitor formation in this region.  
12 These important functions of Hand2 help to define the precise dimensions and  
13 components of the kidneys and vasculature. Moreover, these findings have implications  
14 for understanding the genetic basis of congenital anomalies of the kidney and urinary  
15 tract (CAKUT) and for developing new approaches in regenerative medicine.  
16

## 1 RESULTS

2

### 3 ***hand2* limits pronephron dimensions by repressing pronephron formation**

4 Our interest in the role of *hand2* during kidney development began with an  
5 observation arising from our previously reported microarray analysis of *hand2* mutants  
6 (Garavito-Aguilar et al., 2010). We compared gene expression profiles at 20 hours post  
7 fertilization (hpf) in wild-type embryos and *han<sup>st6</sup>* mutant embryos, which contain a  
8 deletion that removes the entire coding region of *hand2* (Yelon et al., 2000).  
9 Surprisingly, 11 of the 26 transcripts that were increased in *han<sup>st6</sup>* relative to wild-type  
10 were expressed in the pronephron, the embryonic kidney. (For a full list of differentially  
11 expressed genes, see Table S1 in Garavito-Aguilar et al., 2010.) We therefore sought to  
12 understand the effect of *hand2* function on the pronephron.

13 The six genes with the most elevated expression in *han<sup>st6</sup>* mutants are all expressed  
14 in the pronephron tubules. We examined the expression of two of these genes that are  
15 expressed throughout the tubules -- *atp1a1a.4*, which encodes a subunit of the Na<sup>+</sup>/K<sup>+</sup>  
16 ATPase (Thisse et al., 2004), and *cadherin17 (cdh17)* (Horsfield et al., 2002) -- and  
17 observed an increase in tubule width in *han<sup>st6</sup>* (Figure 1A-C, E-G). A similar increase in  
18 width of expression was seen for another gene upregulated in *han<sup>st6</sup>* but only expressed in  
19 a single tubule segment (Wingert et al., 2007), *slc12a3* (Figure 1I-K), which encodes the  
20 thiazide-sensitive sodium-chloride cotransporter solute carrier 12a3. Notably, in contrast  
21 to its widened expression, the anterior-posterior extent of *slc12a3* expression appeared  
22 unaltered. Last, by examining expression of *lhx1a* and *pax2a*, which mark glomerular  
23 precursors at 24 hpf (O'Brien et al., 2011), we found that the populations of glomerular

1 precursors, like the tubules, were expanded in *han*<sup>s6</sup> (Figure 1M-O and Figure 8B,C).

2 Thus, the elevated gene expression detected by microarray analysis in *han*<sup>s6</sup> mutants

3 corresponded with broadened expression of genes throughout the pronephron.

4 To determine if this broadened expression was due to an increase in cell number,

5 we next analyzed the structure of the pronephron in more detail. We found that the

6 number of cells seen in cross-section of the tubule was increased in *han*<sup>s6</sup> (Figure 1Q-S).

7 This increase in cell number was observed at multiple regions along the anterior-posterior

8 axis, suggesting that the entire pronephron is expanded. Concomitant with this increase

9 in the number of cells, the total tubule area in cross-section was increased in *han*<sup>s6</sup>

10 mutants (Figure 1T). Together with the expanded expression of pronephron genes, these

11 findings implicate *hand2* in inhibiting the accumulation of pronephron cells.

12 Does *hand2* simply prevent excessive expansion of the pronephron or is it potent

13 enough to repress pronephron formation? To differentiate between these possibilities, we

14 examined the effects of *hand2* overexpression on the pronephron. Injection of *hand2*

15 mRNA inhibited pronephron formation, as assessed by *atp1a1a.4* expression (Figure

16 1D). To bypass any potential effects of overexpression on gastrulation, we also utilized

17 our previously characterized *Tg(hsp70:FLAG-hand2-2A-mCherry)* transgenic line to

18 drive *hand2* expression at the tailbud stage (Schindler et al., 2014). Heat shock-induced

19 overexpression resulted in pronephron defects comparable to those caused by mRNA

20 injection, as assessed by *atp1a1a.4*, *cdh17* and *slc12a3* expression (Figure 1H,L and

21 Figure 3A,B). Furthermore, like tubule gene expression, *lhx1a* and *pax2a* expression in

22 glomerular precursors was dramatically inhibited by *hand2* overexpression (Figure 1P

23 and data not shown), again emphasizing the broad effect of *hand2* function on

1 pronephron formation. In contrast to these effects of *hand2* overexpression on the  
2 pronephron, most other regions of expression of these markers, such as expression of  
3 *atp1a1a.4* in mucus-secreting cells and expression of *lhx1a* in spinal neurons, were  
4 unaffected (Figure 1D,P). Thus, the effect of *hand2* overexpression seems to reflect its  
5 particular impact on pronephron development, as opposed to a general influence of *hand2*  
6 on the expression of each of these genes. Of note, however, we did observe a dramatic  
7 reduction in otic vesicle expression of both *atp1a1a.4* and *pax2a* (data not shown),  
8 suggesting a shared susceptibility to *hand2* overexpression in the otic vesicles and the  
9 pronephron. Taken together, our loss-of-function and gain-of-function studies show that  
10 *hand2* is necessary to constrain the size of the pronephron, likely through an ability to  
11 repress pronephron formation.

12

### 13 ***hand2* limits intermediate mesoderm dimensions by repressing intermediate** 14 **mesoderm formation**

15 We next sought to determine the origin of the effect of *hand2* on the pronephron,  
16 and we hypothesized that the pronephron defects observed in *han<sup>sf</sup>* mutants might reflect  
17 a requirement for *hand2* to limit IM dimensions. Indeed, using two established IM  
18 markers, *lhx1a* and *pax2a*, we observed that the width of the IM was expanded in *han<sup>sf</sup>*  
19 mutants and *hand2* morphants (Figure 2A,B and data not shown), at stages shortly after  
20 gastrulation (i.e. at the 6, 10 and 12 somite stages). In contrast, there was no substantial  
21 difference in the length of the IM seen in wild-type and *han<sup>sf</sup>* mutant embryos (data not  
22 shown). Furthermore, compared to wild-type embryos, *han<sup>sf</sup>* mutants typically had ~50%  
23 more Pax2a<sup>+</sup> IM cells (Figure 2D-F). Thus, *hand2* limits the accumulation of IM cells.

1           To determine whether *hand2* is sufficient to repress IM formation, we examined  
2 the effects of *hand2* overexpression. Both *hand2* mRNA injection and heat shock-  
3 induced overexpression of *hand2* resulted in loss of IM, as determined by *pax2a* and  
4 *lhx1a* expression (Figure 2C and data not shown). Taken together with our loss-of-  
5 function analyses, these results suggest that *hand2* limits the dimensions of the IM by  
6 repressing its initial formation.

7           To define the time window during which *hand2* overexpression can inhibit IM  
8 formation, we utilized *Tg(hsp70:FLAG-hand2-2A-mCherry)* embryos to induce *hand2*  
9 expression at different times after gastrulation (Figure 3). Unlike overexpression at the  
10 tailbud, 2 somite, or 6 somite stages (Figure 3A-D), overexpression at the 10 somite stage  
11 failed to inhibit pronephron development (Figure 3E). Furthermore, we found  
12 progressively less severe defects as heat shock induction was performed at successively  
13 later stages between tailbud and 10 somites. For example, while inhibition at the tailbud  
14 or 2 somite stages resulted in the loss of the majority of pronephric *atplala.4* expression  
15 (Figure 3A,B), inhibition at the 6 somite stage resulted in only a mild reduction (Figure  
16 3D). Notably, a common phenotype resulting from overexpression in the later portion of  
17 this timeframe was a slight reduction at the anterior boundary of *atplala.4* expression  
18 (Figure 3D). This finding suggests either that formation of the anterior portion of the  
19 pronephron is the most sensitive to a reduction of IM formation or that this anterior  
20 portion is the last to be formed. Overall, our analysis suggests that there is a *hand2*-  
21 sensitive phase of IM specification prior to the 10 somite stage.

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23

1 ***hand2* is expressed beside the lateral boundary of the intermediate mesoderm**

2       Considering the strong effect of *hand2* function on repressing IM formation, we  
3 sought to define the location of *hand2* expression relative to the IM. Prior studies had  
4 demonstrated bilateral *hand2* expression in the posterior mesoderm of zebrafish, mouse,  
5 chick, and *Xenopus* embryos (Angelo et al., 2000; Fernandez-Teran et al., 2000;  
6 Srivastava et al., 1997; Thomas et al., 1998; Yelon et al., 2000; Yin et al., 2010), but the  
7 precise localization of this expression relative to the IM had not been determined. During  
8 the *hand2*-sensitive phase of IM formation, we found *hand2* to be expressed in bilateral  
9 regions immediately lateral to the IM (Figure 4A,B). At these stages, *hand2* was also  
10 expressed lateral to multiple markers of blood and vessel progenitors, including *etv2*,  
11 *tall1*, and *gata1* (Figure 4C and data not shown). However, a gap lies between these  
12 markers and the *hand2*-expressing cells, consistent with the IM residing between the  
13 lateral *hand2*-expressing cells and the medial blood and vessel progenitors (Figure 4C,D).

14       We also found that the relationship between *hand2* expression, the IM, and blood  
15 and vessel progenitors changed after the completion of the *hand2*-sensitive phase of IM  
16 formation. At approximately the 11 somite stage, a second, lateral population of vessel  
17 progenitors arises at the interface between the IM and the *hand2*-expressing cells (Figure  
18 4F-I). These lateral vessel progenitors are likely to be venous progenitors, based on the  
19 results of a prior study that suggested that the medial, earlier-forming vessel progenitors  
20 contribute to the dorsal aorta and that the lateral, later-forming vessel progenitors  
21 contribute to the primary cardinal vein (Kohli et al., 2013).

22       This set of results defines the general location of *hand2* expression relative to  
23 other territories within the posterior mesoderm (Figure 4E,J). However, these

1 observations do not exclude the possibility of transient overlapping expression at the  
2 boundaries of each territory (e.g. overlapping *etv2* and *pax2a* expression). Furthermore,  
3 we note that gene expression patterns are not necessarily uniform within each territory  
4 (e.g. *tall* and *etv2* expression patterns are neither uniform nor equivalent within their  
5 territory (Kohli et al., 2013)). Nevertheless, our data suggest that, during the *hand2*-  
6 sensitive phase of IM formation, *hand2* may exert its repressive effect on IM formation  
7 by constraining the lateral boundary of the IM. Furthermore, our findings suggest the  
8 possibility of close interactions between *hand2*, the IM and the lateral population of  
9 venous progenitors.

10

### 11 ***hand2* promotes lateral venous progenitor development in the posterior mesoderm**

12 The appearance of lateral venous progenitor cells at the interface between the IM  
13 and the *hand2*-expressing cells raised the question of whether *hand2* regulates the  
14 development of these venous progenitors. To address this possibility, we first assessed  
15 the early expression of *etv2*, *tall*, and *gata1* in the medial population of vessel and blood  
16 progenitors, and we observed no differences between wild-type and *han<sup>s6</sup>* mutant  
17 embryos at either the 6 or 10 somite stages (Figure 5 – Figure Supplement 1 and data not  
18 shown). In contrast, after the 11 somite stage, expression of both *etv2* and *tall* in the  
19 lateral venous progenitor population was absent in *han<sup>s6</sup>* mutants (Figure 5A, B, F, G).  
20 Two-color fluorescent in situ hybridization confirmed that the only territory of *etv2* and  
21 *tall* expression in *hand2* mutants was medial to the IM marker *pax2a* (Figure 5D, E, and  
22 data not shown). Thus, the formation of the lateral venous progenitors, the appearance of

1 which coincides with the end of the *hand2*-responsive phase of IM formation, requires  
2 *hand2*.

3 To gain insight into whether *hand2* directly promotes the formation of vessel  
4 progenitors, we assessed the consequences of *hand2* overexpression. Overexpression of  
5 *hand2* resulted in an expansion of both *etv2* and *tall* expression in the posterior  
6 mesoderm (Figure 5C,H). In contrast, *hand2* overexpression resulted in a reduction of  
7 *gata1* expression (Figure 5K). Thus, in the posterior mesoderm, *hand2* overexpression  
8 can inhibit the development of some tissues, such as IM and blood, while promoting  
9 vessel progenitor formation.

10 To understand the consequences of the influence of *hand2* function on the lateral  
11 venous progenitors, we examined the vasculature in *han<sup>s6</sup>* mutants. As noted above, a  
12 prior lineage tracing study suggested that the lateral venous progenitors contribute to the  
13 posterior cardinal vein (Kohli et al., 2013). Furthermore, this study found that inhibition  
14 of *etv2* function at the time when these lateral cells arise results in loss of expression of  
15 *mannose receptor c1a* (*mrc1a*), a marker of the cardinal vein (Kohli et al., 2013).  
16 Similarly, *han<sup>s6</sup>* mutants lack *mrc1a* and *flt4* expression in the posterior cardinal vein,  
17 suggesting a defect in venous development (Figure 6A,B,D,E). Using *Tg(flk1:ras-*  
18 *mcherry)* to label the entire vasculature, we identified the presence of the posterior  
19 cardinal vein in *han<sup>s6</sup>* mutants (Figure 6J-L), but found that their choroid venous plexus  
20 failed to be properly remodeled (Figure 6M-O). In contrast to these changes in the  
21 venous system, *flt4* and *ephrin-b2a* (*efnb2a*) expression in the dorsal aorta, as well as  
22 *mrc1a* expression in the posterior blood island, appeared grossly unaffected by *hand2*  
23 loss-of-function (Figure 6B,E,H). Meanwhile, consistent with the ability of *hand2* to

1 promote vessel progenitor formation, *hand2* overexpression increased vascular  
2 expression of *mrc1a*, *flt4*, and *efnb2a* (Figure 6C,F,I).

3 Overall, these studies demonstrate a previously unappreciated role for *hand2* in  
4 regulating trunk vasculature development. More specifically, our data indicate that  
5 *hand2* is required for the successful execution of certain aspects of venous differentiation,  
6 including the expression of characteristic venous markers and the remodeling of the  
7 choroid plexus. Combined with prior lineage tracing studies and *etv2* loss-of-function  
8 analysis (Kohli et al., 2013), our findings suggest that *hand2* implements these functions  
9 by promoting the development of the lateral venous progenitors.

10

11 ***hand2* loss-of-function results in increased expression of intermediate mesoderm**  
12 **markers in cells that normally express *hand2***

13 The lateral localization of *hand2* expression and the absence of lateral venous  
14 progenitor markers when *hand2* function is lost highlighted the possibility that *hand2*  
15 impacts the IM at its lateral border. We therefore wanted to determine how the expanded  
16 IM in *han<sup>s6</sup>* mutants relates to the interface between the IM and the *hand2*-expressing  
17 cells. Do the extra IM cells seem to emerge at this interface? Do they appear to arise  
18 from cells that express *hand2*, or, alternatively, at the expense of *hand2*-expressing cells?

19 To differentiate among these possibilities, we used two methods to identify the  
20 *hand2*-expressing territory in *hand2* loss-of-function embryos. We labeled *hand2*-  
21 expressing cells through in situ hybridization in embryos injected with a *hand2*  
22 morpholino (Figure 7A,B), and we took advantage of the non-rescuing BAC transgene  
23 *Tg(hand2:EGFP)* (Kikuchi et al., 2011) to interrogate *han<sup>s6</sup>* mutants (Figure 7C,D). In

1 situ hybridization revealed comparable dimensions of the *hand2* expression territory in  
2 wild-type and morphant embryos (Figure 7A,B). More specifically, using the transgene,  
3 we observed no significant difference between the numbers of GFP<sup>+</sup> cells in the posterior  
4 mesoderm of wild-type and *han<sup>s6</sup>* mutant embryos (Figure 7C-E). Thus, it seems that the  
5 expansion of the IM in *han<sup>s6</sup>* mutants does not come at the expense of the formation of  
6 cells that normally express *hand2*.

7 We next assessed whether the increased number of IM cells in *han<sup>s6</sup>* mutants  
8 could arise at least in part from aberrant IM marker expression in cells that normally  
9 express *hand2*. Careful examination of wild-type embryos carrying the *Tg(hand2:EGFP)*  
10 transgene revealed that ~6% of the GFP<sup>+</sup> cells in their posterior mesoderm also displayed  
11 the IM marker Pax2a (Figure 7C,E). In dramatic contrast, ~60% of the GFP<sup>+</sup> cells in the  
12 *han<sup>s6</sup>* mutant posterior mesoderm were also Pax2a<sup>+</sup> (Figure 7D,E). Intriguingly, the GFP<sup>+</sup>  
13 Pax2a<sup>+</sup> cells were typically observed at or near the border where the *hand2*-expressing  
14 cells meet the IM (Figure 7C,D). These data suggest that *hand2* limits IM dimensions by  
15 constraining the lateral boundary of the IM through repression of IM gene expression  
16 within *hand2*-expressing cells.

17

## 18 **Functionally antagonistic roles for *hand2* and *osr1* in pronephron development**

19 The expansion of Pax2a expression in the *hand2*-expressing territory in *han<sup>s6</sup>*  
20 mutants suggests that factors that promote IM development are present within *hand2*-  
21 expressing cells. We hypothesized that *osr1* could be one of these factors, because of the  
22 correspondence between the *hand2* and *osr1* expression patterns and loss-of-function  
23 phenotypes. First, *osr1* was previously reported to be expressed in the lateral posterior

1 mesoderm, adjacent to the IM (Mudumana et al., 2008). Through direct comparison of  
2 *hand2* and *osr1* expression patterns, we found that the genes are co-expressed in the  
3 lateral posterior mesoderm (Figure 8A). Additionally, prior studies demonstrated that  
4 loss of *osr1* function results in phenotypes opposite to those caused by loss of *hand2*: in  
5 contrast to the expanded pronephron and defective venous vasculature in *han<sup>s6</sup>* mutants,  
6 *osr1* morphants were found to exhibit inhibited pronephron development together with  
7 expansion of venous structures (Mudumana et al., 2008; Tena et al., 2007).

8       To assess the functional relationship between *hand2* and *osr1*, we investigated  
9 double loss-of-function embryos. Dramatically, removal of *hand2* function in the setting  
10 of *osr1* loss-of-function largely restored normal pronephron development. This  
11 interaction was most notable in the glomerular precursor population (Figure 8B-E).  
12 While this population was expanded in *hand2* mutants, it was absent in embryos injected  
13 with an *osr1* morpholino (Figure 8B-D). In *hand2* mutants injected with the *osr1*  
14 morpholino, however, the precursor population appeared comparable to wild-type (Figure  
15 8E). Consistent with prior studies (Mudumana et al., 2008), *osr1* morpholino injection  
16 had a less dramatic effect on the pronephron tubule than on the glomerulus (Figure  
17 8D,H). Nevertheless, a comparable interaction between *hand2* and *osr1* was found in  
18 tubule formation (Figure 8F-I). While *han<sup>s6</sup>* mutants had wide tubules and many *osr1*  
19 morphants had disrupted tubules, *han<sup>s6</sup>* mutants injected with *osr1* morpholino displayed  
20 relatively normal tubules.

21       These data suggest that *hand2* and *osr1* act in opposing and parallel genetic  
22 pathways to regulate the precise dimensions of the pronephron. More generally, our

- 1 findings implicate *hand2* within a genetic network that regulates progenitor cell fates at
- 2 the lateral edge of the IM, at the interface with the *hand2*- and *osr1*-expressing territory.

## 1 **DISCUSSION**

2

3           Together, our data present a new perspective on how the posterior mesoderm is  
4 divided into defined territories that create distinct tissues. Importantly, our work provides  
5 the first evidence that *hand2* restricts the size of the kidney and promotes vein  
6 development. Specifically, our data indicate that *hand2* limits the specification of  
7 pronephric progenitors by restraining the lateral boundary of the IM. Furthermore, at the  
8 end of the *hand2*-sensitive phase of IM formation, *hand2* supports the formation of  
9 venous progenitors just beyond the lateral edge of the IM, in the same region where it  
10 represses IM development. Finally, our data reveal a novel genetic interaction in which  
11 the inhibitory function of *hand2* is balanced by the inductive role of *osr1* during the  
12 establishment of IM dimensions. These findings represent novel functions of Hand2;  
13 although prior studies in mice lacking *Hand2* have examined some posterior structures,  
14 such as the limb buds and enteric nervous system (e.g. Charité et al., 2000; Lei and  
15 Howard, 2011), the influences of Hand2 on the IM and kidney have not been previously  
16 investigated.

17           Our findings highlight the importance of regulating the refinement of the lateral  
18 boundary of the IM. It is intriguing to consider how *hand2*, given its expression in the  
19 lateral posterior mesoderm, could influence the restriction of the adjacent IM. Perhaps  
20 *hand2* acts cell-autonomously to inhibit IM specification; the ectopic appearance of  
21 Pax2a<sup>+</sup> cells within the *hand2*-expressing territory in *hand2* mutants makes this an  
22 attractive model. Alternatively, *hand2* could have a non-autonomous influence on IM  
23 dimensions, perhaps by regulating the production of a lateralizing signal, the absence of

1 which would result in the lateral expansion of IM gene expression. Wherever its precise  
2 location of action may be, this function of *hand2* -- preventing lateral *hand2*<sup>+</sup> *osr1*<sup>+</sup> cells  
3 from acquiring more medial characteristics, such as expression of *pax2a* or *lhx1a* --  
4 suggests a possible mechanism for distinguishing IM gene expression from that of  
5 neighboring lateral territories, a distinct process required during both *in vivo* (James et al.,  
6 2006) and *in vitro* (Takasato et al., 2014) IM development.

7         Additionally, our data imply a close connection between the specification of the  
8 IM and the formation of the lateral venous progenitor population. Are the roles of *hand2*  
9 during IM and venous development coupled to each other, or do these represent two  
10 independent functions of *hand2*? It is appealing to speculate that *hand2* acts to prevent  
11 IM specification within the cells that normally develop into the lateral venous  
12 progenitors. Even so, it remains feasible that *hand2* could have completely separable  
13 effects on the kidney and vein lineages. Currently, the lineage relationships between the  
14 IM, the lateral venous progenitors, and the *hand2*-expressing lateral mesoderm remain  
15 undetermined; future work on the generation of appropriate tissue-specific lineage tracing  
16 tools will facilitate the resolution of these open questions.

17         In future studies, it will also be valuable to elucidate the effector genes that act  
18 downstream of *hand2* in the posterior mesoderm and to determine whether the same set  
19 of effectors mediate its roles during both IM and venous development. It is noteworthy  
20 that our findings have demonstrated a difference between the functions of *hand2* during  
21 vessel progenitor development in the anterior and posterior mesoderm: while we have  
22 previously shown that *hand2* overexpression inhibits the expression of vessel progenitor  
23 markers in the anterior mesoderm (Schindler et al., 2014), here we observed expanded

1 expression of the same markers in the posterior mesoderm of *hand2*-overexpressing  
2 embryos. These distinct responses to *hand2* activity in the anterior and posterior  
3 mesoderm may reflect regional differences in the implementation of different molecular  
4 functions ascribed to Hand2 (Firulli et al., 2005; Funato et al., 2009; Liu et al., 2009;  
5 McFadden et al., 2002; Schindler et al., 2014) or in the utilization of different bHLH  
6 binding partners. Thus, evaluation of the factors that work together with Hand2 in  
7 different embryonic contexts will be crucial for its integration into the genetic regulatory  
8 network responsible for IM and vessel specification.

9 By demonstrating that *hand2* functions in opposition to *osr1*, our data provide the  
10 first key step toward incorporating *hand2* into the genetic regulatory network that  
11 patterns the posterior mesoderm. It is important to note that prior studies have suggested  
12 a non-autonomous role for *osr1* during IM development (Mudumana et al., 2008). The  
13 endoderm is expanded in *osr1* morphants, and mutations that disrupt endoderm  
14 development abrogate the pronephron defects caused by *osr1* loss-of-function  
15 (Mudumana et al., 2008; Terashima et al., 2014). In contrast, we suspect that *hand2*  
16 affects IM formation from its position in the lateral mesoderm, rather than from the  
17 endoderm. We observe ectopic Pax2a<sup>+</sup> cells emerging within *hand2*-expressing  
18 mesoderm in *hand2* mutants; additionally, whereas *osr1* morphants have an expansion of  
19 the endoderm (Mudumana et al., 2008; Terashima et al., 2014), *han*<sup>s6</sup> mutants exhibit  
20 normal amounts of endoderm (Wendl et al., 2007; Yelon et al., 2000). Regardless of  
21 whether *hand2* and *osr1* both function within the lateral posterior mesoderm, our data  
22 suggest that these two genes control antagonistic and parallel genetic pathways that  
23 balance each other's influence in order to regulate the size of the IM.

1 Overall, our new insights into the genetic regulation of IM specification have the  
2 potential to impact our understanding of the origins and treatment of kidney disease.  
3 Prior studies have suggested a genetic component to the origins of congenital anomalies  
4 of the kidney and urinary tract (CAKUT) (Bulum et al., 2013; Vivante et al., 2014). In  
5 some cases of CAKUT, disease-causing mutations have been identified, many of which  
6 disrupt genes that influence early kidney development, including mutations in *PAX2* and  
7 *OSR1* (Madariaga et al., 2013; Thomas et al., 2011; Zhang et al., 2011). By implicating  
8 *hand2* in the control of IM specification, we enrich the gene regulatory network that is  
9 relevant to the etiology of CAKUT. In this regard, it is interesting to note that there are  
10 case reports of chromosomal duplications containing *HAND2* in patients with renal  
11 hypoplasia (Otsuka et al., 2005). Furthermore, the identification of genes that control IM  
12 specification can inspire new directions in the design of stem cell technologies and  
13 regenerative medicine strategies. Alteration of *HAND2* function may allow for  
14 enhancement of current protocols for generating kidney progenitor cells *in vitro* (Kumar  
15 et al., 2015; Mae et al., 2013; Taguchi et al., 2014; Takasato et al., 2014; Takasato et al.,  
16 2015), potentially facilitating the requisite control that will be essential for future  
17 deployment in patient applications.

18

## 1 MATERIALS AND METHODS

2

### 3 Zebrafish

4 We generated embryos by breeding wild-type zebrafish, zebrafish heterozygous  
5 for the *hand2* mutant allele *han<sup>s6</sup>* (Yelon et al., 2000), and zebrafish carrying  
6 *Tg(hand2:EGFP)<sup>pd24</sup>* (Kikuchi et al., 2011), *Tg(hsp70:FLAG-hand2-2A-mCherry)<sup>sd28</sup>*  
7 (Schindler et al., 2014), *Tg(flkl1:ras-mcherry)<sup>s896</sup>* (Chi et al., 2008), or *Tg(etv2:egfp)<sup>ci1</sup>*  
8 (Proulx et al., 2010). For induction of heat shock-regulated expression, embryos were  
9 placed at 37°C for 1 hr and then returned to 28°C. Following heat shock, transgenic  
10 embryos were identified based on mCherry fluorescence; nontransgenic embryos were  
11 analyzed as controls. All heat shocks were performed at tailbud stage unless otherwise  
12 indicated.

13 In embryos older than 20 hpf, *han<sup>s6</sup>* mutants were identified based on their cardiac  
14 phenotype (Yelon et al., 2000). In younger embryos, PCR genotyping of *han<sup>s6</sup>* mutants  
15 was conducted as previously described (Yelon et al., 2000), with the exception of  
16 embryos containing *Tg(hand2:EGFP)<sup>pd24</sup>*, in which we used primers that amplify the first  
17 exon of *hand2* (5'- CCTTCGTACAGCCCTGAATAC-3', 5'-  
18 CCTTCGTACAGCCCTGAATAC-3'). This exon is present in the wild-type allele but is  
19 absent in both *han<sup>s6</sup>* homozygotes and the *TgBAC(hand2:EGFP)<sup>pd24</sup>* transgene.

20

### 21 Injection

22 Synthesis and injection of capped *hand2* mRNA was performed as described  
23 previously (Schindler et al., 2014). To knock down *hand2* function, we injected 3-6 ng

1 of a previously characterized translation-blocking *hand2* morpholino at the one-cell stage  
2 (Reichenbach et al., 2008). To knock down *osr1* function, we used a previously  
3 characterized *osr1* translation-blocking start site morpholino, *osr1* ATG (Tena et al.,  
4 2007). 16-23 ng of this morpholino was injected with or without 1.6 ng of p53  
5 morpholino (Robu et al., 2007).

6

### 7 **In situ hybridization**

8 Standard and fluorescent whole-mount in situ hybridization were performed as  
9 previously described (Brend and Holley, 2009; Thomas et al., 2008), using the following  
10 probes: *atp1a1a.4* (ZDB-GENE-001212-4), *cdh17* (ZDB-GENE-030910-3), *efnb2a*  
11 (ZDB-GENE-990415-67), *etv2* (*etsrp*; ZDB-GENE-050622-14), *flt4* (ZDB-GENE-  
12 980526-326), *gatal* (ZDB-GENE-980536-268), *hand2* (ZDB-GENE-000511-1), *lhx1a*  
13 (*lim1*; ZDB-GENE-980526-347), *mrc1a* (ZDB-GENE-090915-4), *osr1* (ZDB-GENE-  
14 070321-1), *pax2a* (ZDB-GENE-990415-8), *slc12a3* (ZDB-GENE-030131-9505), and  
15 *tall* (*scl*; ZDB-GENE-980526-501). To prepare an *osr1* probe, we amplified and  
16 subcloned the *osr1* cDNA using the primers 5' – GAGTTTCTACCCCGAGTAACCA –  
17 3' and 5' – TTTTCAAAAATAAGTTTAAGGAATCCA – 3'.

18

### 19 **Immunofluorescence and cell counting**

20 Whole-mount immunofluorescence was performed as previously described  
21 (Cooke et al., 2005), using polyclonal antibodies against Pax2a at 1:100 dilution  
22 (Genetex, Irvine, CA, GTX128127) and against GFP at 1:1000 dilution (Life  
23 Technologies, Carlsbad, CA, A10262), and the secondary antibodies goat anti-chick

1 Alexa Fluor 488 (Life Technologies, A11039), goat anti-rabbit Alexa Fluor 594 (Life  
2 Technologies, A11012), and goat anti-rabbit Alexa Fluor 647 (Life Technologies,  
3 A21245), all at 1:100 dilution. Samples were then placed in SlowFade Gold anti-fade  
4 reagent (Life Technologies).

5 To count Pax2a<sup>+</sup> or GFP<sup>+</sup> cells, we flat-mounted and imaged embryos after  
6 dissecting away the yolk and the anterior portion of the embryo. We examined a  
7 representative 250  $\mu$ m long region in roughly the middle of the IM on one side of the  
8 embryo. This technique allowed us to select contiguous regions that were unaffected by  
9 dissection artifacts. Positive cells were determined by examining both three-dimensional  
10 reconstructions and individual optical sections. Statistical analysis of data was performed  
11 using Microsoft Excel to conduct unpaired *t*-tests.

12

### 13 **Histology**

14 Histological analysis was performed on tissues embedded in Spurr Low-Viscosity  
15 embedding mixture. Embryos were fixed in 2% paraformaldehyde and 2.5%  
16 glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), post-fixed with 2% osmium  
17 tetroxide, dehydrated in an ethanol gradient, infiltrated with a propylene oxide/resin  
18 gradient, and then embedded. Samples were oriented as desired and incubated for 24  
19 hours at 60°C for polymerization. 2-4  $\mu$ m sections were cut using glass blades, collected  
20 on standard slides, and stained with 1% toluidine blue.

21 Transverse sections from 24 hpf embryos were examined at three different  
22 anterior-posterior levels along the pronephron, with two sections analyzed per level in  
23 each embryo. We processed three embryos per genotype. Cell counting and area

1 measurements were performed using Zeiss ZEN software. Data variance homogeneity  
2 and normal distribution were confirmed using IBM SPSS Statistics 22 software.

3

#### 4 **Imaging**

5 Bright-field images were captured with a Zeiss AxioCam on a Zeiss Axiozoom  
6 microscope and processed using Zeiss AxioVision. Images of histological sections were  
7 captured using a Zeiss AxioImager A2 microscope coupled to a Zeiss AxioCam camera.  
8 Confocal images were collected by a Leica SP5 confocal laser-scanning microscope and  
9 analyzed using Imaris software (Bitplane).

10

#### 11 **Replicates**

12 All assessments of phenotypes and expression patterns were replicated in at least  
13 two independent experiments with comparable results. Embryos were collected from  
14 independent crosses, and experimental processing (injection, heat shock, and/or staining)  
15 was carried out on independent occasions. Two exceptions to this include data presented  
16 in Figure 1Q-T and Figure 8C-E. In each of those cases, multiple embryos were  
17 processed, and the n is reported in the associated figure legends.

18

19

1 **ACKNOWLEDGEMENTS**

2 We thank members of the Yelon lab, N. Chi, L. Oxburgh, and D. Traver for valuable  
3 discussions; I. Drummond, K. Poss, S. Sumanas, J. Torres-Vázquez, and D. Traver for  
4 providing reagents; L. Rincón-Camacho with assistance with histology; and H. Knight  
5 for assistance with graphics.

6

7 **COMPETING INTERESTS**

8 All authors declare no competing interests.

9

10 **AUTHOR CONTRIBUTIONS**

11 EAP: Conception and design, acquisition of data, analysis and interpretation of data,  
12 writing the manuscript. ZVGA: Conception and design, acquisition of data, analysis and  
13 interpretation of data. GPGV and KTP: Acquisition of data. YLS: Contributed  
14 unpublished reagents. DY: Conception and design, analysis and interpretation of data,  
15 writing the manuscript.

16

17 **FUNDING**

18 This work was supported by grants to DY from the National Institutes of Health (NIH)  
19 [R01HL069594 and R01HL108599] and from the March of Dimes [1-FY16-257]. EAP  
20 was supported by California Institute of Regenerative Medicine Training Grant TG2-  
21 01154 and by an A. P. Giannini Foundation Fellowship. ZVGA was supported by the  
22 Universidad de los Andes Assistant Professor Fund (FAPA) and by the Vicerrectoría de

- 1 Investigaciones P14.160422.007/01. KTP was supported by the Colciencias
- 2 Convocatoria 617-2013-Joven Investigador Fellowship.
- 3

1 **FIGURE LEGENDS**

2

3 **Figure 1. *hand2* inhibits pronephron formation.**

4 (A-P) Dorsal views, anterior to the left, of pronephron schematics (A, E, I, M), wild-type  
5 embryos (B, F, J, N), *han<sup>s6</sup>* mutant embryos (C, G, K, O), and *hand2*-overexpressing  
6 embryos (D, injected with *hand2* mRNA; H, L, P, carrying *Tg(hsp70-hand2-2A-*  
7 *mcherry)*, abbreviated *hs:hand2*) at 24 hpf. In schematics (A, E, I, M), colored regions  
8 correspond to area of pronephron gene expression. In situ hybridization demonstrates  
9 that *atp1a1a.4* (A-D) and *cdh17* (E-H) are expressed throughout the pronephron tubules,  
10 *slc12a3* (I-L) is expressed in the distal late segments of the pronephron tubules, and *lhx1a*  
11 (M-P) is expressed in the glomerular precursors (arrows, N), as well as overlying spinal  
12 neurons (asterisks, N). Compared to wild-type (B, F, J, N), gene expression is expanded  
13 in *han<sup>s6</sup>* mutants (C, G, K, O) and reduced in *hand2*-overexpressing embryos (D, H, L, P).  
14 Of note, injection of a *hand2* translation-blocking morpholino caused effects on  
15 pronephron formation similar to those seen in *han<sup>s6</sup>* mutants (data not shown). Scale bars  
16 represent 100  $\mu$ m.  
17 (Q, R) Transverse sections through wild-type (Q) and *han<sup>s6</sup>* mutant (R) pronephron  
18 tubules at 24 hpf. Dashed lines outline the tubule and asterisks indicate individual tubule  
19 cells.  
20 (S, T) Bar graphs indicate the average number of tubule cells per cross-section (S) and  
21 the average tubule area per cross-section (T) in wild-type and *han<sup>s6</sup>* mutant embryos;  
22 error bars indicate standard error. Asterisks indicate statistically significant differences  
23 compared to wild-type ( $p < 0.0001$ , Student's t test;  $n = 18$ ).

1 **Figure 2. *hand2* inhibits IM production.**

2 (A-C) Dorsal views, anterior to the top, of the posterior mesoderm at the 10 somite stage.

3 In situ hybridization depicts normal expression of *lhx1a* in the IM (arrows) of wild-type  
4 embryos (A), widened expression in *han<sup>s6</sup>* mutants (B), and reduced expression in *hand2*-  
5 overexpressing embryos (C). Expression in the notochord (asterisk) is unaffected by  
6 altered *hand2* function. Scale bar represents 100  $\mu$ m.

7 (D-E) Pax2a immunofluorescence in the posterior mesoderm of wild-type (D) and *han<sup>s6</sup>*  
8 mutant (E) embryos at the 12 somite stage. Dorsal views, anterior to the left, are three-  
9 dimensional reconstructions of flat-mounted embryos from which the yolk and anterior  
10 tissues have been dissected away. Scale bar represents 100  $\mu$ m.

11 (D'-E') Magnification of 250  $\mu$ m long regions from (D) and (E) used for quantification of  
12 the number of Pax2a<sup>+</sup> cells in wild-type (D') and *han<sup>s6</sup>* mutant (E') embryos. White dots  
13 indicate Pax2a<sup>+</sup> nuclei. Intensity of staining varied from strong (for example, green  
14 arrows) to weak (for example, yellow arrows). Scale bar represents 25  $\mu$ m.

15 (F) Bar graph indicates the average number of Pax2a<sup>+</sup> cells in a 250  $\mu$ m long portion of  
16 the IM in wild-type and *han<sup>s6</sup>* mutant embryos; error bars indicate standard error.

17 Asterisk indicates a statistically significant difference compared to wild-type ( $p < 0.0001$ ,  
18 Student's t test;  $n = 13$  for wild-type and  $n = 10$  for *han<sup>s6</sup>*).

19

1 **Figure 3. Pronephron development is susceptible to *hand2* overexpression prior to**  
2 **the 10 somite stage.**

3 (A-E) Dorsal views, anterior to the left, of in situ hybridization for *atp1a1a.4* at 24 hpf  
4 depict a range of severity of pronephron defects, ranging from absence of the pronephron  
5 (A) to unaffected (E). *Tg(hsp70:FLAG-hand2-2A-mCherry)* embryos were subjected to  
6 heat shock at the tailbud, 2 somite, 6 somite, or 10 somite stages, and the consequences  
7 on pronephron development were scored at 24 hpf. Percentages indicate the distribution  
8 of phenotypes produced by each treatment; the number of embryos examined is in the  
9 right-hand column. Heat shock at later stages resulted in more mild loss of *atp1a1a.4*  
10 expression in the tubule, and heat shock at the 10 somite stage did not disrupt *atp1a1a.4*  
11 expression in the tubule. Scale bar represents 100  $\mu$ m.

12

1 **Figure 4. *hand2* expression in the posterior lateral mesoderm.**

2 (A-D, F-I) Two-color fluorescent in situ hybridization (A, C, F, H) and  
3 immunofluorescence (B, D, G, I) label components of the posterior mesoderm in dorsal  
4 views, anterior to the left, of three-dimensional reconstructions, as in Figure 2D-E. In  
5 embryos containing transgenes in which GFP expression is driven by the regulatory  
6 elements of *hand2* (B, G) or *etv2* (D, I), anti-GFP immunofluorescence was used to  
7 enhance visualization. *Tg(etv2:egfp)* expression was also observed in the midline neural  
8 tube, as previously reported (Proulx et al., 2010). Scale bar represents 100  $\mu$ m.  
9 (E, J) Schematics depict posterior mesoderm territories, dorsal views, anterior to the left;  
10 *hand2*-expressing cells, IM, and medial vessel/blood progenitors are shown at 2-10  
11 somites (E) and at 11-13 somites (J), together with lateral vessel progenitors.  
12 (A-D) *hand2* is expressed lateral to the IM at the 2-10 somite stages. Embryos shown are  
13 at the 10 somite stage; similar expression patterns were seen at earlier stages. (A, B)  
14 *hand2* is expressed lateral to the IM markers *lhx1a* (A) and Pax2a (B). (C) *hand2* is  
15 expressed lateral to *tall*, a marker of blood and vessel progenitors; note the unlabeled gap  
16 between expression territories. (D) A marker of vessel progenitors, *Tg(etv2:egfp)*, lies  
17 medially adjacent to Pax2a.  
18 (F-I) Vessel progenitors arise at the interface between *hand2*-expressing cells and the IM  
19 at the 11-13 somite stages. (F, G) *hand2* is expressed lateral to the IM marker *pax2a*.  
20 (H) *hand2* is expressed lateral to a second territory of *tall* expression; note the presence  
21 of lateral *tall*-expressing cells (arrows) immediately adjacent to *hand2* expression. (I)  
22 The IM lies between two territories of *etv2* expression; the more lateral *etv2*-expressing  
23 cells (arrows) are lateral to the IM.

1 **Figure 5. *hand2* promotes vessel progenitor development.**

2 (A-C, F-K) In situ hybridization depicts *etv2* (A-C), *tall* (F-H) and *gatal* (I-K)  
3 expression in wild-type (A, F, I), *han<sup>s6</sup>* mutant (B, G, J) and *hand2*-overexpressing (C, H,  
4 K) embryos; dorsal views, anterior to the left, at the 12 somite stage. (A, F) *etv2* and *tall*  
5 are expressed in relatively medial and lateral (arrows) territories on each side of the wild-  
6 type embryo. In *han<sup>s6</sup>* embryos (B, G), only the medial territory is present. In *hand2*-  
7 overexpressing embryos (C, H), expression of both *etv2* and *tall* is increased, but it is not  
8 possible to distinguish whether this represents an increase in the medial or the lateral  
9 territories. (I-K) *gatal* expression is equivalent in wild-type (I) and *han<sup>s6</sup>* (J) embryos,  
10 but it is decreased in *hand2*-overexpressing embryos (K). Scale bar represents 100  $\mu$ m.  
11 (D, E) Fluorescent in situ hybridization depicts relationship of *etv2* and *pax2a* expression  
12 in wild-type (D, D') and *hand2* morphant (*hand2* MO) embryos (E, E'); dorsal views,  
13 anterior to the left, at the 12 somite stage. Medial and lateral (arrows) territories of *etv2*  
14 expression flank *pax2a* in wild-type embryos (D, D'). The lateral territory of expression  
15 is absent in *hand2* morphants (E, E').  
16

1 **Figure 5 – Figure Supplement 1. Presence of medial vessel and blood progenitors is**  
2 **unaffected in *han*<sup>s6</sup> mutants.**  
3 (A-F) In situ hybridization depicts expression of *etv2* (A, B), *tall* (C, D), and *gata1* (E, F)  
4 in wild-type (A, C, E) and *han*<sup>s6</sup> mutant (B, D, F) embryos; dorsal views, anterior to the  
5 top, of the posterior mesoderm at the 6 somite stage. Scale bar represents 100 μm.  
6

1 **Figure 6. *hand2* promotes proper vein formation.**

2 (A-I) In situ hybridization depicts expression of *mrc1a* (A-C), *flt4* (D-F) and *efnb2a* (G-I)  
3 in wild-type (A, D, G), *han<sup>st6</sup>* mutant (B, E, H) and *hand2*-overexpressing (C, F, I)  
4 embryos; lateral views, anterior to the left, at 24 hpf (A-C, G-I) and the 20 somite stage  
5 (D-F).

6 (A-C) *mrc1a* expression in the posterior cardinal vein (blue arrow) was present in wild-  
7 type (A), absent in *han<sup>st6</sup>* mutant (B), and increased in *hand2*-overexpressing (C) embryos.  
8 Expression in the posterior blood island (red arrowhead) was grossly unaffected.

9 (D-F) *flt4* expression in the posterior cardinal vein (blue arrow) was present in wild-type  
10 (D), absent in *han<sup>st6</sup>* mutant (E), and increased in *hand2*-overexpressing (F) embryos.  
11 Expression in the dorsal aorta (red arrow) was grossly unaffected.

12 (G-I) *efnb2a* expression in the dorsal aorta (red arrow) was present in wild-type (G),  
13 grossly unaffected in *han<sup>st6</sup>* mutant (H), and slightly increased in *hand2*-overexpressing (I)  
14 embryos.

15 (J-O) Lateral views of three-dimensional reconstructions of *Tg(flk1:ras-mcherry)*  
16 expression in the vasculature of wild-type (J, M) and *han<sup>st6</sup>* mutant (K, N) embryos at 28  
17 hpf (J, K) and 32 hpf (M, N). Pictured region in (J, K) is the area of the trunk boxed in  
18 the schematic (L); pictured region in (M, N) is the area of the tail boxed in the schematic  
19 (O). Both posterior cardinal vein (blue arrow) and dorsal aorta (red arrow) were present  
20 in wild-type (J) and *han<sup>st6</sup>* mutant (K) embryos. In contrast to wild-type (M), the caudal  
21 venous plexus (arrowhead) fails to undergo proper remodeling in *han<sup>st6</sup>* mutants (N).

22 Scale bars represent 100  $\mu$ m.

23

1 **Figure 7. Increased presence of Pax2a in *hand2*-expressing cells of *hand2* mutants.**

2 (A-B) In situ hybridization depicts presence of *hand2* expression in *hand2* morphants  
3 (B); dorsal views, anterior to the left, at the 10 somite stage. There is no evident loss of  
4 *hand2*-expressing cells in *hand2* morphants; moreover, *hand2* expression levels appear  
5 higher in the context of *hand2* loss-of-function. Scale bar represents 100  $\mu\text{m}$ .

6 (C-D) Immunofluorescence for Pax2a and GFP in wild-type (C) and *han<sup>s6</sup>* mutant (D)  
7 embryos, both carrying *Tg(hand2:EGFP)*; dorsal views, anterior to the left, of three-  
8 dimensional reconstructions at the 12 somite stage, as in Figure 4G.

9 (C'-D'') Magnification of 250  $\mu\text{m}$  long regions from (C) and (D) used for quantification  
10 of the numbers of GFP<sup>+</sup> and Pax2a<sup>+</sup> cells in wild-type and *han<sup>s6</sup>* mutant embryos. Yellow  
11 dots indicate GFP<sup>+</sup> cells, white dots indicate Pax2a<sup>+</sup> nuclei, and examples of GFP<sup>+</sup> Pax2a<sup>+</sup>  
12 cells are indicated by white arrows. Intensity of Pax2a<sup>+</sup> staining varied from strong (for  
13 example, green arrows) to weak (for example, yellow arrows). Scale bars represent 100  
14  $\mu\text{m}$  (C, D) and 25  $\mu\text{m}$  (C'-D'').

15 (E) Bar graph indicates the average numbers of GFP<sup>+</sup> cells and GFP<sup>+</sup> Pax2a<sup>+</sup> cells in a  
16 250  $\mu\text{m}$  long region in wild-type and *han<sup>s6</sup>* mutant embryos; error bars indicate standard  
17 error. Asterisk indicates a statistically significant difference compared to wild-type  
18 ( $p < 0.0001$ , Student's t test;  $n = 13$  for wild-type and  $n = 10$  for *han<sup>s6</sup>*).

19

1 **Figure 8. *hand2* and *osr1* act in opposing, parallel pathways to regulate pronephron**  
2 **development.**

3 (A-A") Fluorescent in situ hybridization depicts overlap (A) of *hand2* (A') and *osr1* (A")  
4 expression in wild-type embryos; dorsal views, anterior to the left, of three-dimensional  
5 reconstructions at the 6 somite stage.

6 (B-I) In situ hybridization depicts *pax2a* (B-E) and *atp1a1a.4* (F-I) expression at 24 hpf  
7 in wild-type embryos (B, F), *han<sup>s6</sup>* mutant embryos (C, G), *osr1* morphant (*osr1* MO)  
8 embryos (D, H) and *han<sup>s6</sup>* mutant embryos injected with *osr1* morpholino (*han<sup>s6</sup>* + *osr1*  
9 MO) (E, I); dorsal views, anterior to the left.

10 (B-E) Compared to wild-type (B), *pax2a* expression in the glomerular and neck  
11 precursors was expanded in 100% of *han<sup>s6</sup>* mutants (C, n=11), absent (48%) or reduced  
12 (48%) in *osr1* morphants (D, n=25), and relatively normal in *han<sup>s6</sup>* + *osr1* MO embryos  
13 (E, n=11). While the extent of marker expression was generally comparable to wild-type  
14 in the double loss-of-function embryos, the stereotypic patterning of this population was  
15 often somewhat disrupted.

16 (F-I) Compared to wild-type (F), *atp1a1a.4* expression in the pronephric tubules was  
17 wide in 85% of *han<sup>s6</sup>* mutants (G, n=13), while many *osr1* morphants (H, n=133) had  
18 tubules with shortened anterior expression (18%) or tubules with segmental losses (35%),  
19 and 47% of *osr1* morphants had a wild-type appearance. 85% of *han<sup>s6</sup>* + *osr1* MO  
20 embryos (I, n=46) resembled wild-type, whereas 11% had a shortened anterior tubule and  
21 4% had segmental losses in the tubules.

22 Scale bars represent 100  $\mu$ m.

23

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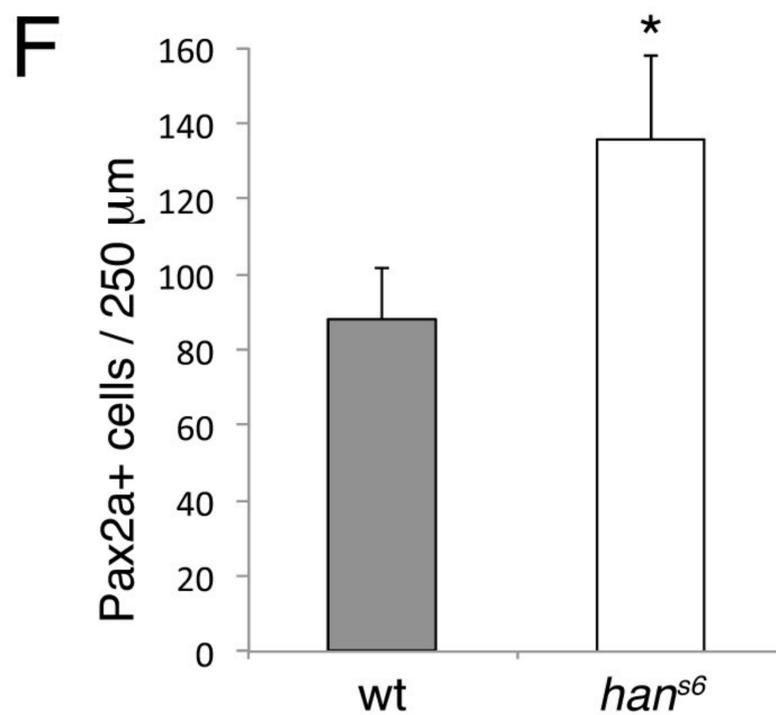
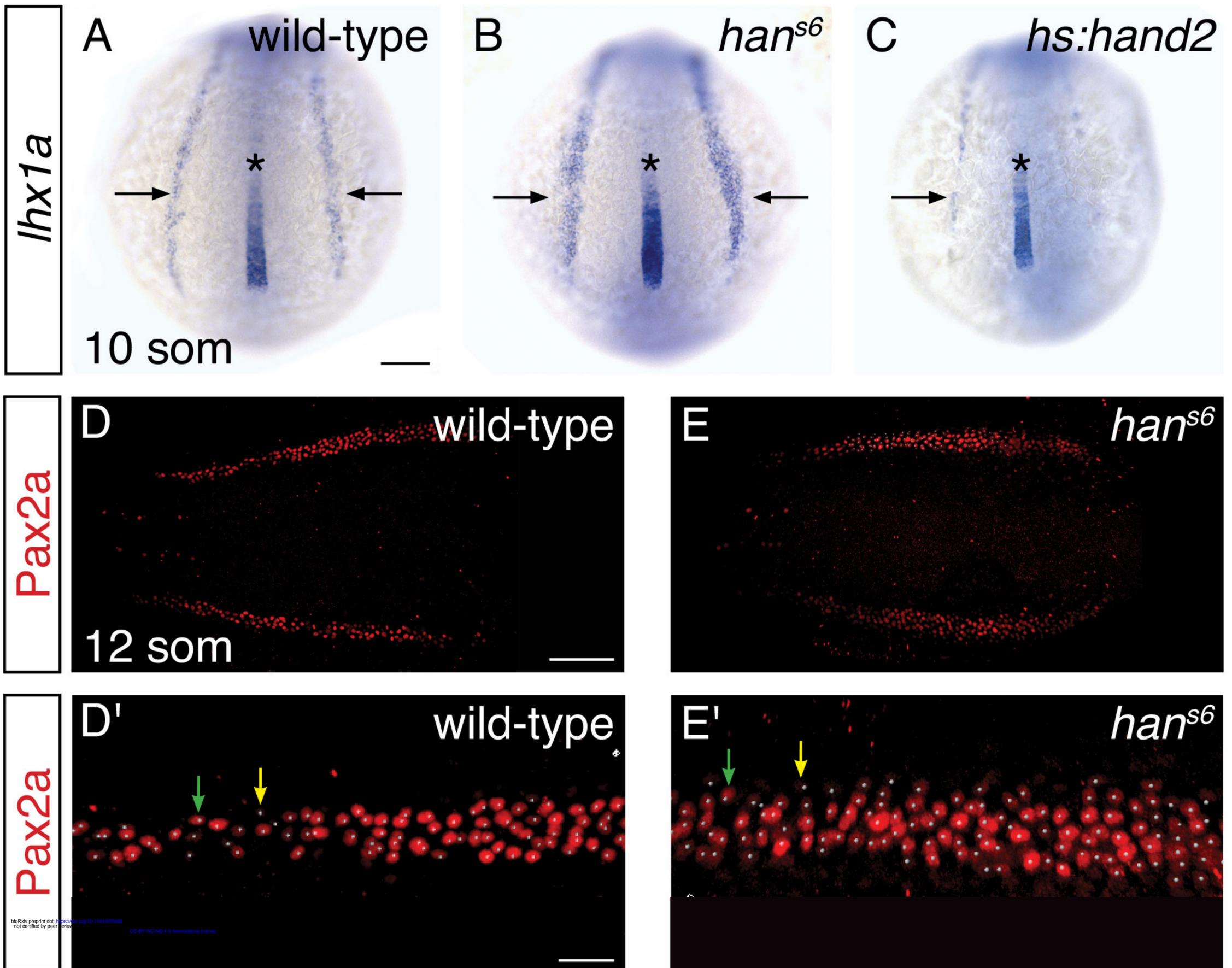
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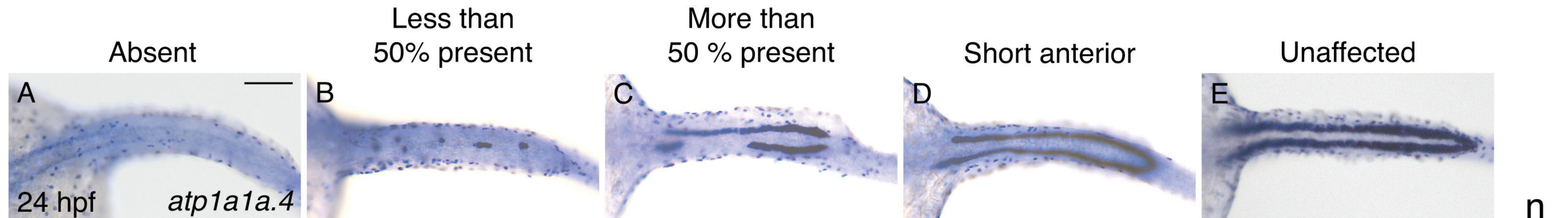
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- 8
- 9



FIGURE 2



**FIGURE 3**



|                          | Absent | Less than 50% present | More than 50 % present | Short anterior | Unaffected | n  |
|--------------------------|--------|-----------------------|------------------------|----------------|------------|----|
| Heat shock at tailbud    | 35%    | 55%                   | 10%                    | -              | -          | 20 |
| Heat shock at 2 somites  | -      | 25%                   | 20%                    | 55%            | -          | 20 |
| Heat shock at 6 somites  | -      | -                     | -                      | 50%            | 50%        | 20 |
| Heat shock at 10 somites | -      | -                     | -                      | -              | 100%       | 20 |

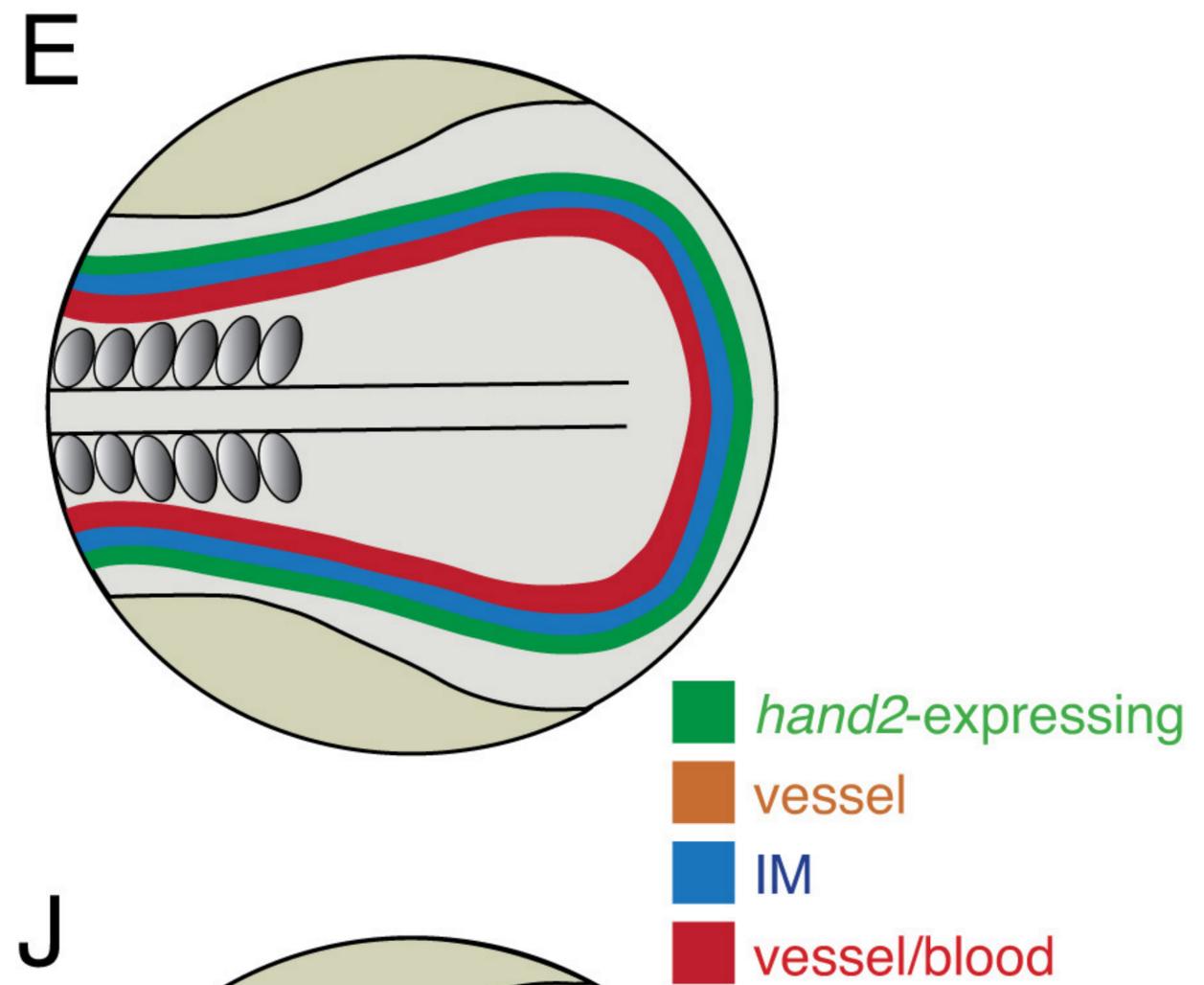
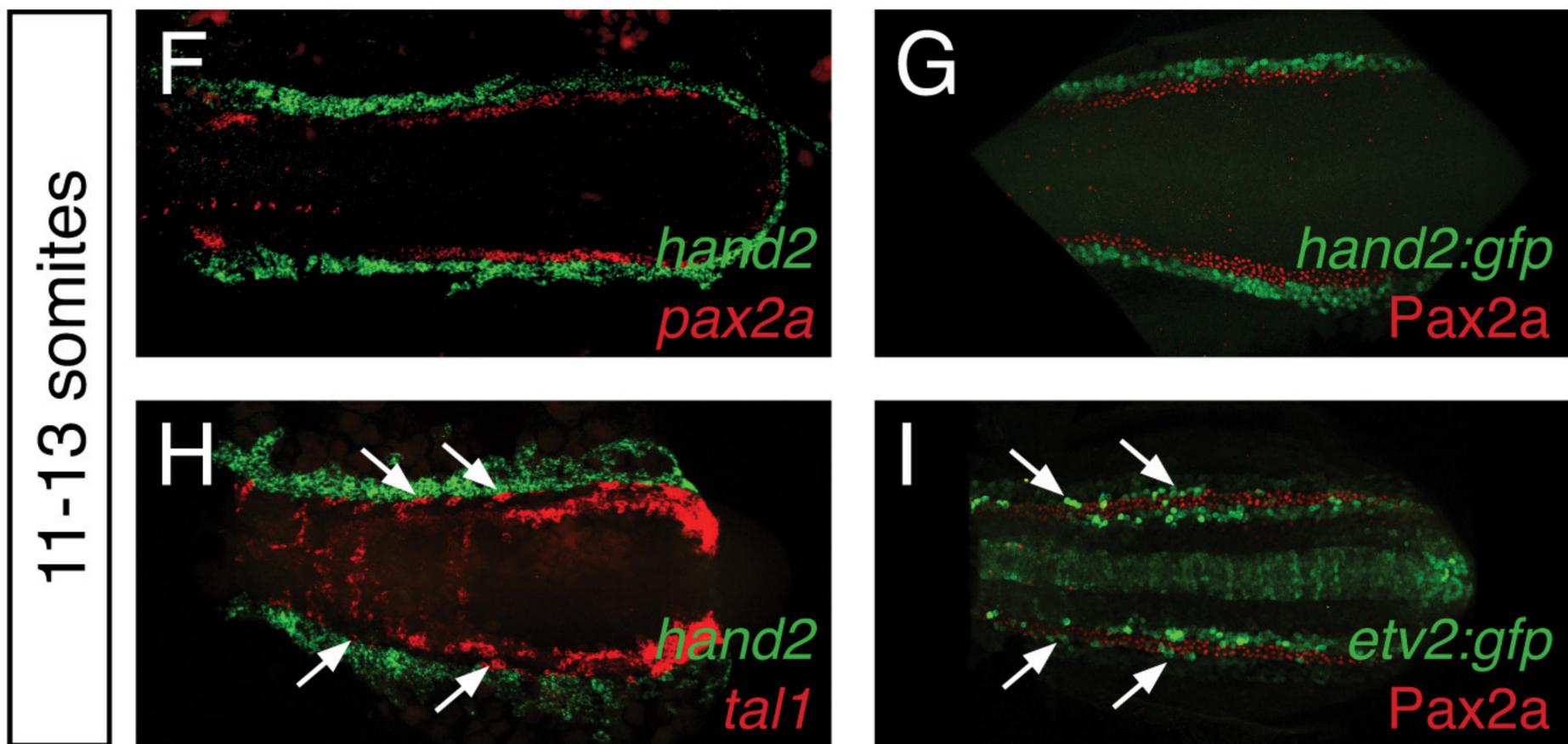
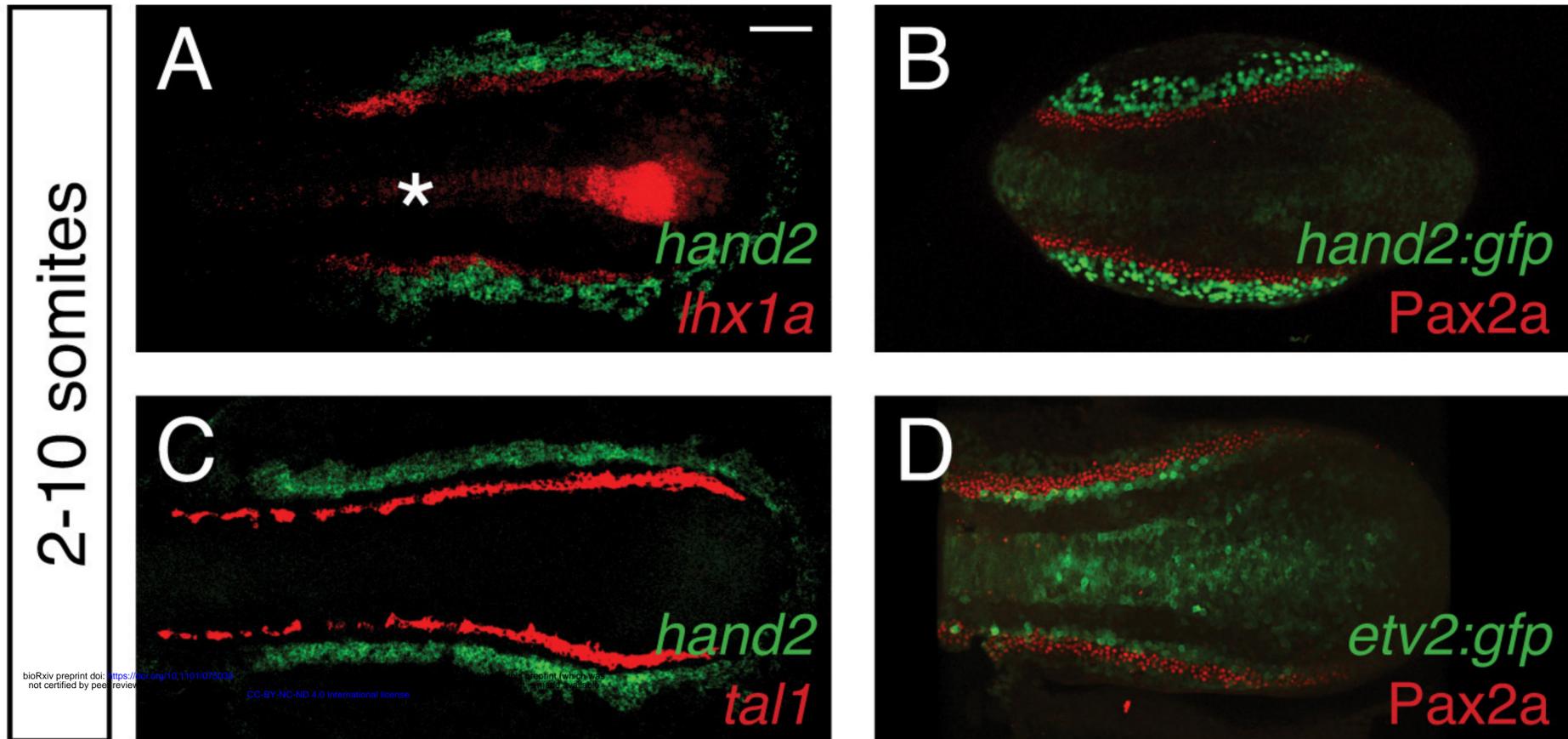
**FIGURE 4**

FIGURE 5

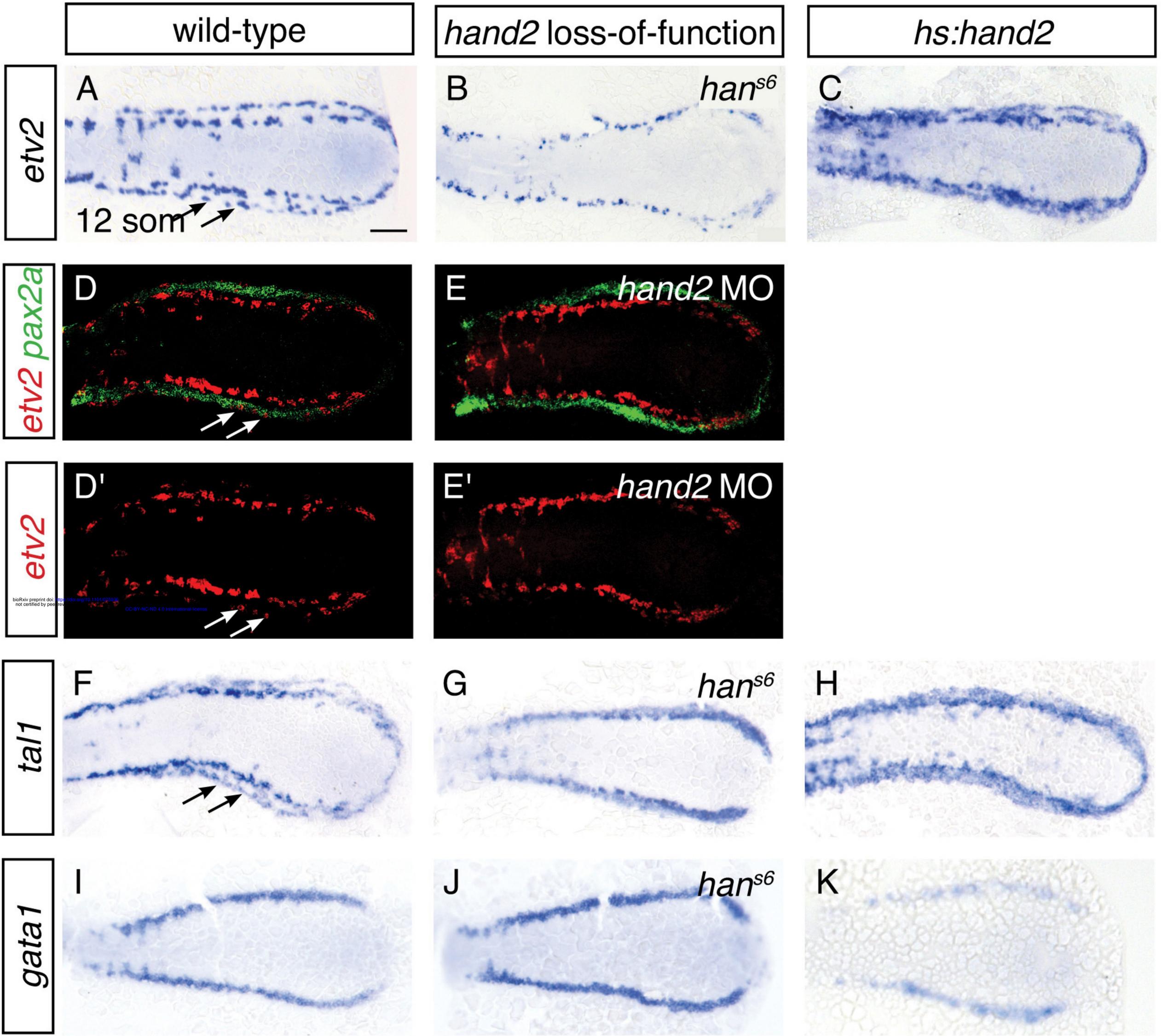


FIGURE 5 - FIGURE SUPPLEMENT 1

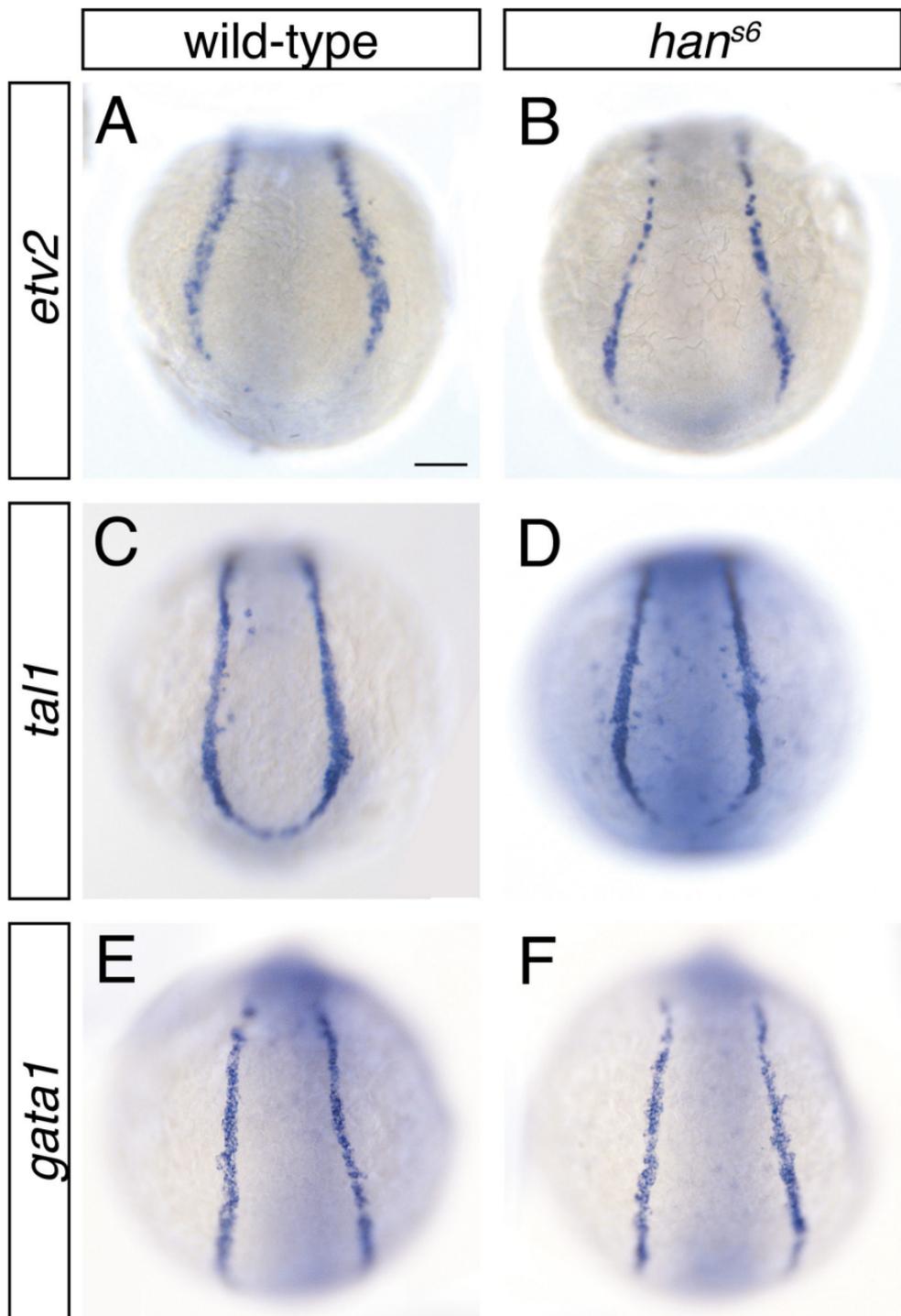


FIGURE 6

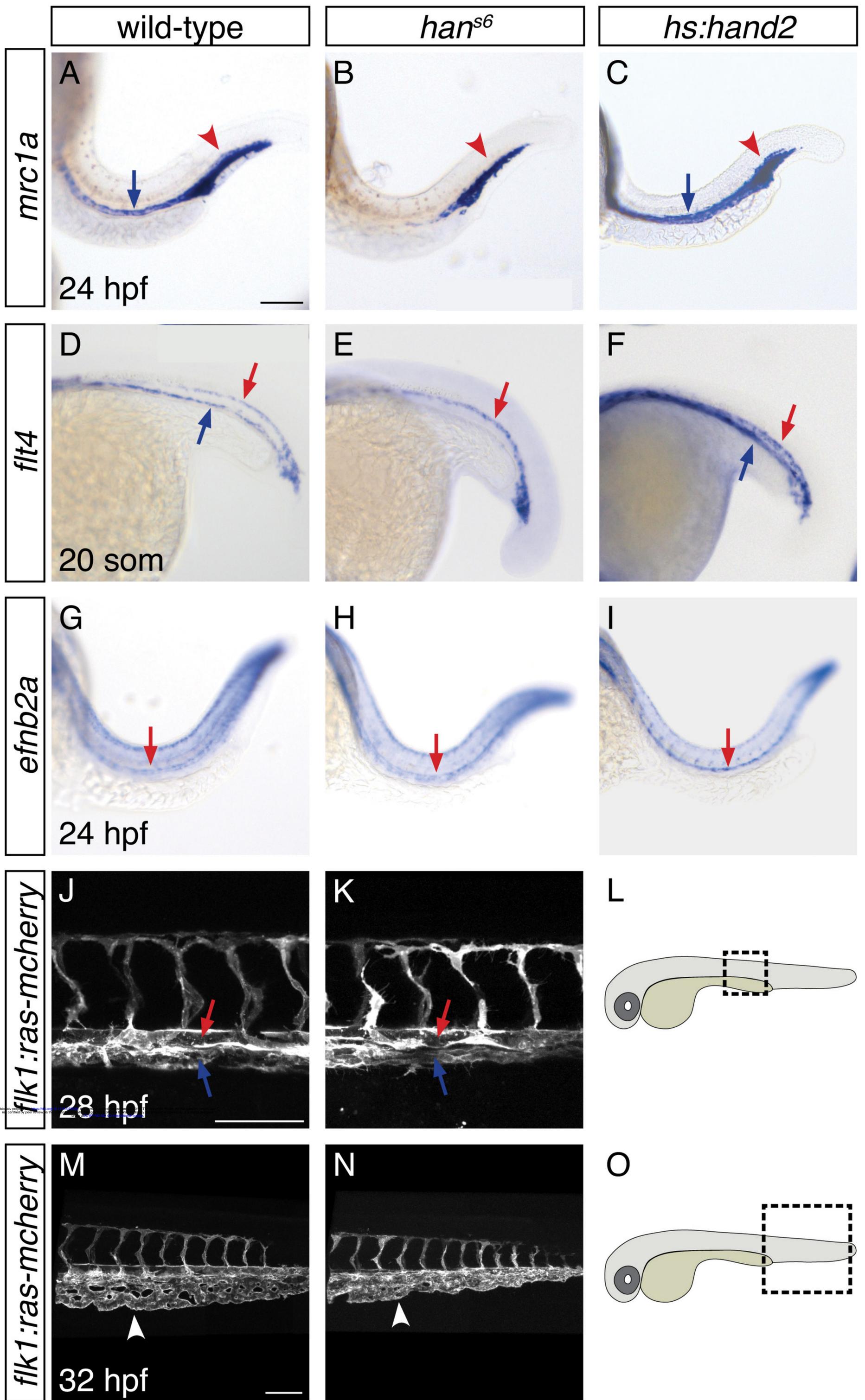


FIGURE 7

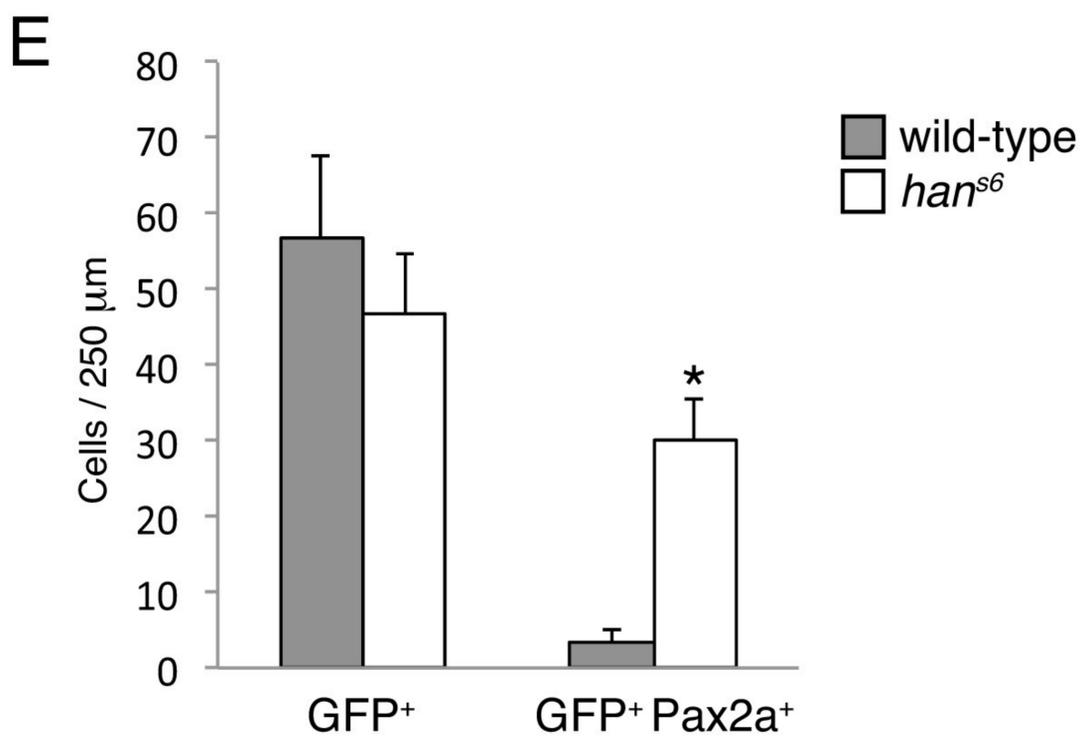
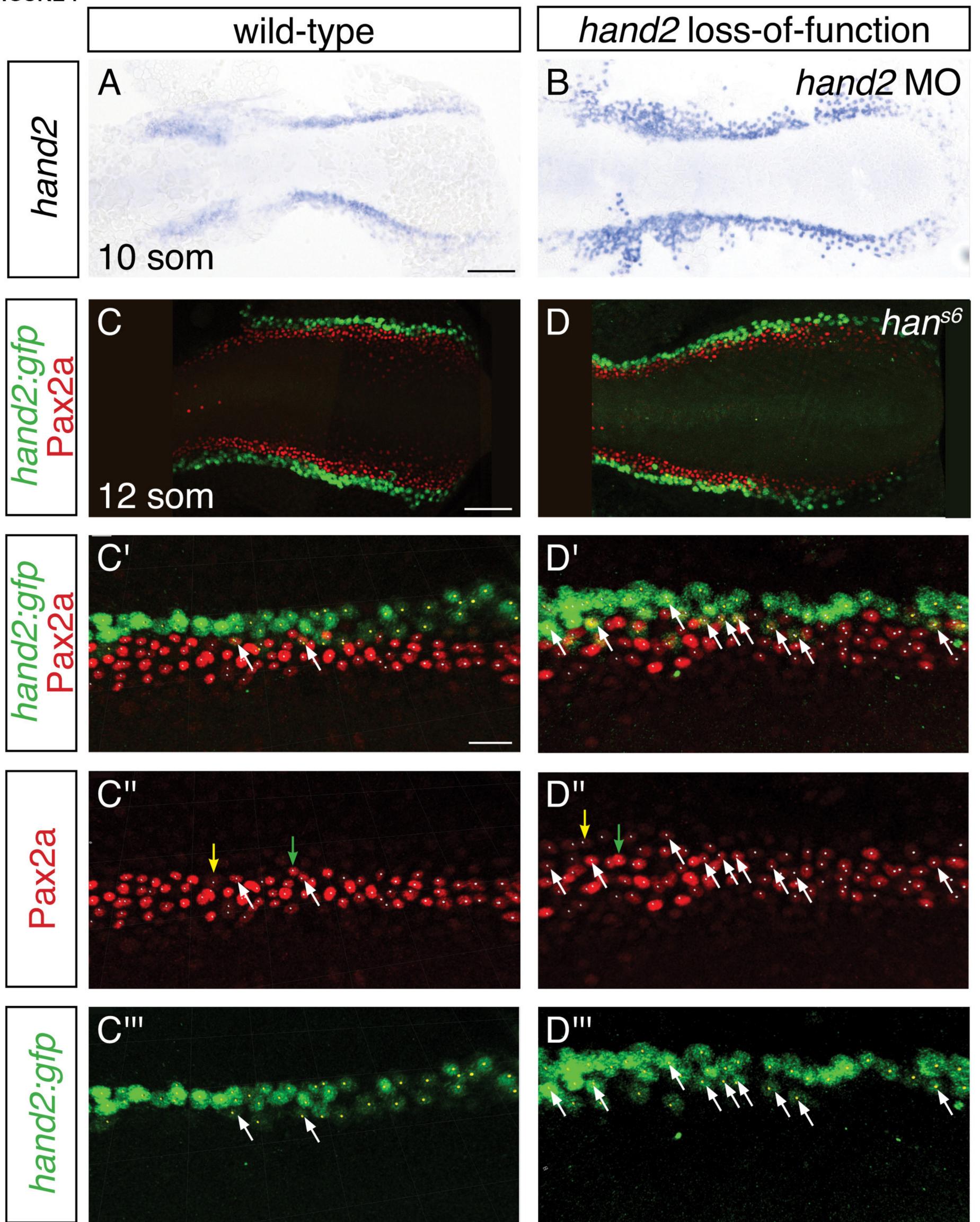


FIGURE 8

