Transcription Factors with Broad Expression in the Zebrafish Spinal Cord

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Abstract

Background

The spinal cord is a crucial part of the vertebrate central nervous system, controlling movements and receiving and processing sensory information from the trunk and limbs. However, there is much we do not know about how this essential organ develops. Here, we describe expression of 22 transcription factor genes in the zebrafish spinal cord.

Results

We analyzed the spinal cord expression of *aurkb, foxb1a, foxb1b, her8a, homeza, ivns1abpb, mybl2b, myt1a, nr2f1b, onecut1*, *sall1a, sall3a, sall3b, sall4, sox2*, *sox19b, sp8b, tsc22d1, wdhd1*, *zfhx3b, znf804a*, and *znf1032* in wild-type and *MIB E3 ubiquitin protein ligase 1* zebrafish embryos. While all of these genes are broadly expressed in the spinal cord, they have distinct expression patterns from one another. Some are predominanatly expressed in progenitor domains, and others in subsets of post-mitotic cells. Given the conservation of spinal cord development, and the transcription factors that regulate it, we expect that these genes will have similar spinal cord expression patterns in other vertebrates, including mammals and humans.

Conclusions

Our data identify 22 different transcription factors that are strong candidates for playing different roles in spinal cord development. For several of these genes, this is the first published description of their spinal cord expression.

Introduction

The spinal cord is a crucial part of the Central Nervous System (CNS), responsible for controlling movements and receiving and processing sensory information from the trunk and the limbs. Even though the spinal cord is relatively simple compared to the brain, there are still fundamental gaps in our knowledge of how it develops and appropriate neuronal and glial spinal cell types are made, maintained and connected into appropriate neuronal circuitry. Transcription factors play crucial roles in these processes, helping to regulate co-ordinated programs of gene expression [1-3]. However, we have no idea how many transcription factors are expressed in the spinal cord during development and the temporo-spatial expression patterns of many transcription factors are currently unknown. This significantly impedes our ability to identify the regulatory gene networks that orchestrate spinal cord development. To begin addressing this, as part of a larger gene expression screen to identify transcription factor genes that are expressed in the developing spinal cord [4,5] we identified 22 genes that are expressed broadly in the zebrafish embryonic spinal cord. When we started this study, the expression of many of these genes was unknown. Since then, some of them have been studied by other groups, although often in other tissues. In most cases, the published expression data is still limited, particularly in the spinal cord and for some of the genes that we analyze in this paper, there are currently no other published reports of their expression in zebrafish embryos. For example, expression of *znf1032*, has, to our knowledge, never been examined in any vertebrate before. There is also no published zebrafish expression data for *homeza, ivns1abpb, mybl2b, onecut1, tsc22d1* or *wdhd1*, although there are some online photographs available from large scale expression screens [6-12].

In this report, we describe the spinal cord expression of all 22 of these genes in zebrafish embryos at key developmental stages, and we also show their expression in the brain and other trunk tissues. In addition, we examine whether these genes are expressed in progenitor and/or post-mitotic spinal cells. We analyze their expression in wild-type embryos in both whole-mount preparations and lateral cross-sections and in *MIB E3 ubiquitin protein ligase 1* (*mib1*, formerly called *mind bomb*) mutants. *mib1* encodes a ubiquitin ligase that is part of the Notch pathway, and in *mib1*^{ta52b} mutants the vast majority of spinal cord progenitor cells precociously differentiate as early-forming populations of spinal cord post-mitotic neurons, at the expense of later forming neurons and glia [13-17]. This enables us to distinguish between progenitor domain expression (which should be lost

in *mib1* mutants) and post-mitotic expression (which is often, although not always, expanded in *mib1* mutants). In combination with analysing wild-type trunk cross-sections, this allows us to discriminate between expression in progenitor cells and post-mitotic cells. Interestingly, we find that while all of these genes are broadly expressed in the spinal cord, they have distinct expression patterns from one another. Some are expressed in progenitor cells, others in both progenitor cells and different subsets of post-mitotic cells, and others are predominantly expressed in distinct subtypes of post-mitotic cells. This identification of transcription factor genes with distinct expression patterns in progenitor and/or post-mitotic cells is an essential first step in determining the gene regulatory networks that specify the correct development of spinal cord circuitry and function.

Results

We have performed several different high-throughput gene expression screens to identify transcription factor genes that are expressed in the developing spinal cord [e.g. 4,5]. In previous studies, we analyzed transcription factors that are expressed by specific types of spinal cord interneurons [4,5,18-23]. However, we have also identified a subset of 22 transcription factor genes that our initial analyses suggested were broadly expressed in the zebrafish embryonic spinal cord, but are not ubiquitious throughout the embryo. As discussed in the introduction, spinal cord expression of some of these genes has not been reported before, while for most of the others, there are only limited descriptions of their expression in the published literature (see Discussion for more detail).

As these genes may play important roles in spinal cord development, we analyzed their expression in more detail. At 24 hours post fertilization (h), *aurkb, foxb1a, foxb1b, her8a, homeza, ivns1abpb, mybl2b, myt1a, nr2f1b, onecut1*, *sall1a, sall3a, sall3b, sall4, sox2*, *sox19b, sp8b, tsc22d1, wdhd1*, *zfhx3b, znf804a*, and *znf1032* are all expressed throughout the rostro-caudal and dorso-ventral spinal cord (Fig. 1). All of these genes are also broadly expressed throughout the hindbrain (Figs. 1, 4, 5 and 6, for a schematic of anatomical locations see Fig. 1W) and all of them except *znf804a* and *znf1032* (Fig. 1U, V) have distinct, spatially-restricted expression patterns within the fore- and midbrain. In contrast, *znf804a* and *znf1032* are more broadly expressed in these brain regions (Figs. 1 and 5). In addition, *aurkb*, *ivns1abpb*, *mybl2b*, *sall4*, *wdhd1*, *znf804a*, and *znf1032* are variably expressed in the blood (located in the dorsal aorta and cardinal vein) beneath the notochord (Fig. 1A, F, G, N, S, U, V, W).

Analyses of spinal cord cross-sections show that most of these genes, *aurkb, foxb1a, foxb1b*, *her8a, homeza*, *ivns1abpb, mybl2b, nr2f1b, sall1a, sall3b*, *sall4, sox2, sox19b, sp8b, tsc22d1, wdhd1, zfhx3b, znf804a,* and *znf1032*, are expressed in both the medial (progenitor), and lateral (post-mitotic) domains of the spinal cord (Fig. 2). These genes are also expressed throughout the dorso-ventral axis of the spinal cord, except for *sall3b* and *sp8b*, which are only expressed in the ventral two-thirds of the spinal cord (Fig. 2M, Q), and *foxb1b* and *wdhd1*, which are expressed throughout the dorso-ventral medial (progenitor) domains of the spinal cord, but are only expressed in the ventral two-thirds of the lateral (post-mitotic) domains of the spinal cord (Fig. 2C, S). In contrast,

myt1a and *onecut1* are only expressed in the lateral (post-mitotic) domains of the spinal cord (Fig. 2H, J), and if *sall3a* is expressed in the medial spinal cord, that expression is much weaker than the lateral expression (Fig. 2L). *myt1a* and *onecut1* expression extends to all dorso-ventral domains of the spinal cord, but *sall3a* expression is absent in the dorsal-most spinal cord (Fig. 2H, J, L). Consistent with the whole-mount analyses (Fig. 1), *aurkb*, *ivns1abpb*, *mybl2b*, *sall4*, *wdhd1*, *znf804a*, and *znf1032* expression is also visible in the blood immediately beneath the notochord (Fig. 2A, F, G, N, S, U, V, W). In addition, *sox2* is also expressed in the hypochord, at the ventral interface between the notochord and the blood (Fig. 2O, for a schematic of anatomical locations see Fig. 2W) and *tsc22d1* is expressed in the pronephros, which are tubular structures paired ventrally, lateral to the blood, on each side of the cross-section (Fig. 2R, and see Fig. 2W).

The spinal cord expression of *aurkb, foxb1a, homeza, myt1a, onecut1, sall1a, sall3b, sall4, sox2, sox19b, sp8b*, *zfhx3b*, and *znf1032* persists at 36 h (Fig. 3A, B, E, H, J, K, M-Q, T, V). In contrast, the spinal cord expression of *foxb1b, her8a, ivns1abpb, mybl2b, nr2f1b, sall3a, tsc22d1, wdhd1*, and *znf804a* decreases by this stage, either persisting most strongly in ventral spinal cord (*foxb1b*, *her8a*, *ivns1abpb*, *mybl2b*, *sall3a*, and *znf804a*), only very weakly in the spinal cord (*nr2f1b* and *tsc22d1*), or only in very few spinal cells (*wdhd1*) (Fig. 3C, D, F, G, I, L, R, S, U). *her8a* and *sox2* are now also expressed in neuromasts, deposited at intervals along the length of the embryo by the migrating lateral line primordium (black arrows, Fig. 3W, X).

To further confirm whether these genes are expressed in progenitor cells or post-mitotic cells, we also examined their expression in 24 h *mib1*^{ta52b} mutants. As described in the introduction, the vast majority of spinal cord progenitor cells precociously differentiate as early-forming populations of neurons in these mutants, at the expense of later forming neurons and glia [13-17]. Therefore, spinal cord progenitor domain expression is lost and post-mitotic expression is often, although not always, expanded in these mutants.

We found that expression of five of the genes, *aurkb, foxb1b, her8a, wdhd1* and *zfhx3b,* is lost from the spinal cord in all but the very caudal tail in *mib1*^{ta52b} mutants (Fig. 4), suggesting that these genes are all expressed in spinal cord progenitor cells. Interestingly though, brain expression of these genes is largely unchanged in these mutants, with the exception that *her8a* expression is

slightly less intense, and *zfhx3b* expression is slightly expanded in the telencephalon (Fig. 4M, W). *aurkb* and *wdhd* expression in the blood is also unaffected (Fig. 4E, T).

Eleven other genes also lose a lot of their normal spinal cord expression in *mib1*^{ta52b} mutants, but in contrast to the five genes discussed above, they are still expressed in distinct subsets of spinal cord cells (Fig. 5). Expression of *mybl2b, nr2f1b, sox19b*, and *foxb1a* is lost in all but a few dorsal spinal cord cells, whereas expression of *homeza* and *ivns1abpb* persists in more cells but only in dorso-caudal spinal cord (Fig. 5D, H, L, P, T, X). In contrast, small clusters of cells in both dorsal and ventral spinal cord still express *sall4, sox2*, *sp8b*, and *znf1032*, whereas *znf804a* is only expressed in small clusters of ventral spinal cord cells (Fig. 5B', F', J', N', R'). These data suggest that these genes are expressed broadly in spinal cord progenitor cells, and also in a small number of post-mitotic spinal cord cells.

Interestingly, and in contrast to the five genes discussed above (Fig. 4), all eleven of these genes also have a dramatic reduction in hindbrain expression in *mib1*^{ta52b} mutants. However, consistent with the five genes discussed above, their expression in the fore- and midbrain regions is largely unchanged in *mib1*^{ta52b} mutants, with the exception of *sall4*, which is slightly expanded in the dorsal midbrain, and *sox2* and *sp8b*, which are almost lost in the dorsal forebrain (Fig. 5A', E', I'). Expression of *mybl2b*, *ivns1abpb*, *sall4*, *znf1032*, and *znf804a* in the blood is unaffected in *mib1*^{ta52b} mutants (Fig. 5D, X, B', N', R').

Unlike the 16 genes discussed above, expression of the remaining six genes, *myt1a, onecut1, sall1a, sall3a, sall3b*, and *tsc22d1*, persists and is expanded in the spinal cord of *mib1*^{ta52b} mutant embryos (Fig. 6). This suggests that these genes are expressed by post-mitotic spinal cord cells. *myt1a*, *onecut1*, *sall1a*, and *sall3a* expression is also expanded throughout the brain in *mib1*^{ta52b} mutants, including the hindbrain (Fig. 6C, G, K, O). In contrast, *sall3b* and *tsc22d1* expression is only expanded in the hindbrain and largely unchanged elsewhere in the brain (Fig. 6S, W).

Discussion and Conclusions

In this paper we describe the expression of 22 different transcription factor genes in the zebrafish spinal cord. Given the high conservation of spinal cord development and transcription factor expression in vertebrates [24,25], these data are relevant not only to zebrafish development, but also to other vertebrates, including mammals and humans. All of these genes have broad, but not ubiquitious, expression patterns in the spinal cord. This suggests that they have specific functions in particular subsets of spinal cells, rather than general house-keeping roles in all cells. In order to hypothesize about the possible functions of these genes, we first need to know whether they are expressed in progenitor and/or post-mitotic cells in the spinal cord. At the stages that we examined, spinal cord progenitor cells are located medially and as cells become post-mitotic, they move laterally. In addition, spinal cord progenitor domain expression is lost and post-mitotic expression is usually expanded in *mib1*^{ta52b} mutants. Based on our cross-sectional analyses and the dramatic reduction of their spinal cord expression in *mib1*^{ta52b} mutants, we conclude that *aurkb, foxb1b, her8a, wdhd1*, and *zfhx3b* are all expressed in spinal cord progenitor cells (Figs. 2 and 4). *mybl2b, nr2f1b, sox19b*, *foxb1a, homeza*, *ivns1abpb, sall4, sox2*, *sp8b*, *znf1032* and *znf804a* are also predominantly expressed in progenitor cells, but *mybl2b, nr2f1b, sox19b*, *foxb1a, homeza* and *ivns1abpb* are also expressed in some dorsal post-mitotic cells, whereas *sall4, sox2*, *sp8b*, and *znf1032* are expressed in some post-mitotic cells in both the dorsal and ventral spinal cord and *znf804a* is expressed in only some ventral spinal cord post-mitotic cells (Figs. 2 and 5). In contrast, *myt1a, onecut1, sall1a, sall3a, sall3b*, and *tsc22d1* are predominantly expressed by different subsets of post-mitotic spinal cord cells, although our cross-sectional analyses suggest that *sall1a, sall3b* and *tsc22d1* are also expressed in spinal cord progenitor cells (Figs. 2 and 6).

This is the first detailed description of spinal cord expression for most of these genes, in any vertebrate. The exceptions are *her8a* [26,27], *sall1a* [28], *sall3a* [28], *sox2* [29-32], *sox19b* [30,33-35] and *sp8b* [36,37]. There is also some limited spinal expression data at 24 h for *aurkb* [38,39 supplementary data], *foxb1b* [40,41], *nr2f1b* [42 supplementary data], *sall4* [43], *zfhx3b* [44], as well as at stages earlier than those we examine in this paper for *foxb1a* [40], *foxb1b* [45], *myt1a* [46] and *sall1a* [28]. Finally, there are some data at www.ZFIN.org or https://ibcs-bip-web1.ibcs.kit.edu/ffdb/ from large scale zebrafish expression screens, that show spinal cord expression of *aurkb*, *foxb1a, foxb1b, her8a, homeza, ivns1abpb, mybl2b, nr2f1b, onecut1, sall1a, sall3b, sall4, sox2, sox19b, sp8b, tsc22d1, wdhd1* and *zfhx3b* [6-11].

For *homeza, ivns1abpb, mybl2b, onecut1, tsc22d1,* and *wdhd1,* these online database photos are the only spinal cord expression data that we are aware of, outside this paper. To our knowledge, there is no other expression data, in any tissue, in any vertebrate, for *znf1032* and no spinal cord expression data in any vertebrate for *znf804a*, not even in online databases of large-scale expression screens.

For all but one of the genes where there is some additional spinal cord expression data, our results are consistent with these other reports. The one possible exception is *sall3b*, where a photo from a large scale expression screen shows unrestricted expression, although no lateral views are provided so it is possible that this experiment just had higher levels of background expression than our data [7]. However, for several genes, the data from the other sources shows weaker / less apparent spinal cord expression than our results and / or does not show spinal cord expression at stages older than 24 h. This is probably because many of these studies were not concentrating specifically on the spinal cord and, therefore, developed their *in situ* hybridization staining reactions to a level more appropriate for examining expression in other tissues. Spinal cord expression is often weaker than, for example, brain expression, particularly at later stages [22,23].

Notably, even in cases where some expression in the spinal cord has previously been shown, with the exception of *sall1a* [28], *sall3a* [28], *sox2* [32,47] and *sp8b* [37] there are no cross-sectional analyses of spinal expression and, with the exception of *her8a* [48], there is no analysis of spinal cord expression in *mib1* mutants, which means that it is often not clear whether the reported spinal cord expression is in progenitor and / or post-mitotic cells. This is a crucial piece of information for considering the functional roles of these genes in the spinal cord. In this paper, we have analyzed these aspects of expression for all 22 genes.

Interestingly, as described above, we find that while all of the genes that we examined are broadly expressed in the spinal cord, most of them have distinct expression patterns from one another. This suggests that they have different, specific functions in the spinal cord. However, for most of these genes, there is currently no data to suggest what their function(s) may be in the spinal cord. The exceptions are *aurkb,* which may be important for axonal outgrowth of spinal motor neurons [49], *her8a,* which is important for Notch signaling and, hence, neurogenesis e.g. [27,48,50], *onecut1,* which

data from mouse and chick suggest may be important for motoneuron and V1 interneuron (Renshaw cell) development [51-53], *sox2,* which is required for correct differentiation of spinal motoneurons and oligodendrocytes [32,54,55] and *sp8b,* which is required for correct specification of the pMN/p3 progenitor domain boundary and hence the correct development of the cells that develop from these domains [37,56]. Interestingly, at earlier stages of spinal cord development, *sall4* and *sp8b*, might belong to a common gene regulatory network as *sall4* is thought to regulate *pou5f3* expression, which in turn regulates *hoxb1a/b* expression in the posterior neurectoderm that forms the spinal cord, and *hoxb1a/b* over-expression can induce expression of *sp8b* [57-60]. Further eludidation of these functions and the roles of the other genes in spinal cord development await further studies.

In conclusion, this study identifies 22 transcription factors with specific spinal cord expression patterns, that may have important roles in spinal cord development. In this way, it provides key new knowledge for Developmental Biologists, especially those interested in CNS development, and / or specific transcription factors or gene regulatory networks.

Experimental Procedures

Ethics statement

All zebrafish experiments in this research were carried out in accordance with the recommendations and approval of Syracuse University Institutional Animal Care and Use (IACUC) committee.

Zebrafish husbandry and fish lines

Zebrafish (*Danio rerio*) were maintained on a 14-h light/10-h dark cycle at 28.5°C. Embryos were obtained from natural paired and/or grouped spawnings of wild-type (WT; AB, TL or AB/TL hybrid) fish, or heterozygous *mib1*^{ta52b} mutants [14].

in situ hybridization

We fixed embryos in 4% paraformaldehyde / phosphate-buffered saline (PBS) and performed single *in situ* hybridization experiments as previously described [17,61]. We synthesized *in situ* RNA riboprobes using the methods described in [18] using the primers and annealing temperatures shown in Table 1. To avoid cross-reactivity, whenever possible, riboprobes were designed against 3'UTR or coding sequence lacking all conserved protein domains in Pfam [62]. Primers were designed using Primer3 web version 4.1.0 at https://primer3.ut.ee [63,64] and the following design parameters: optimum primer size: 22 bp (minimum: 20 bp, maximum: 25 bp), optimum annealing temperature: 58.0ºC (minimum: 57.0ºC, maximum: 60.0ºC), and optimum GC content: 50% (minimum: 40%, maximum: 60%). The preferred product size range was 800-1100 bp. This was not always possible, if there was little or no novel coding and/or 3' UTR sequence available (see Table 1). The PCR conditions were: 98.0ºC for 30 seconds, 35 cycles of: 98.0ºC for 10 seconds; annealing temperature in Table 1 for 30 seconds and 72.0ºC for 30 seconds, followed by a final extension for 5 minutes at 72.0ºC. The PCR product was assessed on a 1% TAE gel, before purifying through phenol:chloroform:isoamyl alcohol extraction and precipitation with 0.2 M NaCl and ice-cold ethanol. If non-specific banding was generated in addition to the desired PCR product, the specific product was purified from the agarose gel using the Monarch DNA Gel Extraction Kit (NEB, T1020S). Each reverse primer contains the T3 RNA Polymerase minimal promoter sequence (shown in bold and underlined in Table 1). *in situ* probe synthesis was performed using 1 µg

purified PCR product, T3 RNA Polymerase (11031171001, Roche) and DIG RNA Labeling Mix (11277073910, Roche).

Embryos older than 24 h were usually incubated in 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigment formation. For some experiments, we added 5% of Dextran Sulfate to the hybridization buffer. In cases where this was done it is indicated in the relevant figure legend. Dextran sulfate can increase specific staining in *in situ* hybridization experiments as it facilitates molecular crowding [65,66].

In cases of low riboprobe hybridization efficiency, we exposed embryos to prolonged staining. In some cases, this produced higher background (diffuse, non-specific staining), especially in the hindbrain, where ventricles can sometimes trap anti-sense riboprobes [23].

Imaging

All embryos were deyolked in 70% glycerol / 30% sterile water using mounting pins. For lateral and dorsal views of the embryo, whole embryos were mounted in 70% glycerol between coverslip sandwiches (24 mm x 60 mm coverslips; VWR, 48393-106), with 2-4 coverslips (22 mm x 22 mm; VWR, 16004-094) on either side of the sample to avoid sample compression. Cross-sections were cut by hand using a razor blade mounted in a 12 cm blade holder (World Precision Instruments, Cat. # 14134). Differential interference contrast (DIC) pictures were taken using an AxioCam MRc5 camera mounted on a Zeiss Axio Imager M1 compound microscope. All images were processed for brightness-contrast and colour balance using Adobe Photoshop software [67]. Images of control and *mib1*^{ta52b} mutant embryos are from the same experiment and they were processed identically. Figures were assembled using Adobe Photoshop [67].

Author Contributions

S.J.E. designed the primers for all *in situ* hybridization RNA probes. S.J.E., P.C.C., and G.G. performed *in situ* hybridization RNA riboprobe synthesis. P.C.C. performed the majority of *in situ* hybridization experiments, with additional assistance from S.J.E., R.L.B., G.G., and W.F.F. R.L.B. performed most of the embryo cross-sectioning experiments, with additional help from S.J.E. S.J.E. performed the majority of imaging, with additional assistance from P.C.C., R.L.B., and W.F.F. S.J.E. performed much of the data analysis and made all figures and tables in the paper. K.E.L. conceptualised and directed the study, acquired the financial support for the project, contributed to data analysis, and wrote the paper. All authors read and commented on drafts of the paper and approved the final version.

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Figure Legends

Figure 1. Broad Expression of Transcription Factor Genes in Wholemount Zebrafish Embryos at 24 h.

(A-V) Lateral views of wholemount wild-type zebrafish embryos at 24 h. (W) Schematic of a lateral view of a 24h wholemount zebrafish embryo. F=forebrain, M=midbrain, H = hindbrain V = mid- and hindbrain ventricles, SC = spinal cord, N = notochord, B = blood (black dotted line indicates boundary between dorsal aorta and cardinal vein), Y = yolk and the eye and lens are indicated with concentric blue dotted circles. In all panels, rostral is left and dorsal is up. Transcription factor genes (A) *aurkb,* (B) *foxb1a,* (C) *foxb1b,* (D) *her8a,* (E) *homeza,* (F) *ivns1abpb,* (G) *mybl2b,* (H) *myt1a,* (I) *nr2f1b,* (J) *onecut1*, (K) *sall1a,* (L) *sall3a,* (M) *sall3b,* (N) *sall4,* (O) *sox2* (P), *sox19b,* (Q) *sp8b,* (R) *tsc22d1,* (S) *wdhd1*, (T) *zfhx3b,* (U) *znf804a*, and (V) *znf1032* are all broadly expressed throughout the rostro-caudal and dorso-ventral spinal cord and all 22 of these genes are also variably expressed in the brain. (A) *aurkb*, (F) *ivns1abpb*, (G) *mybl2b*, (N) *sall4*, (S) *wdhd1*, (U) *znf804a*, and (V) *znf1032* are also variably expressed in the blood beneath the notochord. (N) *sall4 in situ* hybridization experiments were performed with the molecular crowding reagent Dextran Sulfate (see Experimental Procedures for rationale). All other *in situ* hybridization experiments in this figure were performed without this reagent. Scale bar: 200 µm.

Figure 2. Broad Expression of Transcription Factor Genes in Zebrafish Spinal Cord at 24 h.

(A-V) Cross-section views of trunk expression of transcription factor genes (A) *aurkb,* (B) *foxb1a,* (C) *foxb1b*, (D) *her8a,* (E) *homeza*, (F) *ivns1abpb,* (G) *mybl2b,* (H) *myt1a,* (I) *nr2f1b,* (J) *onecut1,* (K) *sall1a,* (L) *sall3a,* (M) *sall3b*, (N) *sall4,* (O) *sox2,* (P) *sox19b,* (Q) *sp8b,* (R) *tsc22d1,* (S) *wdhd1,* (T) *zfhx3b,* (U) *znf804a,* and (V) *znf1032* in wild-type zebrafish embryos at 24 h. Dorsal, up. As indicated in the schematic cross-section (W), the spinal cord (SC), is located above the notochord (N), which is above the hypochord (H, indicated with red arrow), dorsal aorta (DA), and the cardinal vein (CV). The somites (S) can be seen on both sides of these tissues and the pronephros tubes (P, indicated with red arrows) are ventral, either side of the cardinal vein. Wtihin the spinal cord, the dotted line indicates the midline, the small oval indicates the central canal and the small black triangles indicate the roof plate and floor plate. (N) *sall4 in situ* hybridization

experiments were performed with the molecular crowding reagent Dextran Sulfate (see Experimental Procedures for rationale). All other *in situ* hybridization experiments in this figure were performed without this reagent. Scale bar: 30 µm.

Figure 3. Transcription Factor Gene Expression in Zebrafish Embryos at 36 h.

Lateral views of spinal cord (A-V) or neuromasts of the lateral line primordium (W, X) in wild-type zebrafish embryos at 36 h. Rostral, left. Dorsal, up. In the spinal cord views, the morphological boundary between the ventral spinal cord and the notochord is visible towards the bottom of the panel and the spinal cord is in focus. In panels (W, X) the focal plane is more lateral, and the somites and lateral line primordium are in focus. Black arrows (W, X) indicate neuromasts, deposited at intervals along the length of the embryo by the migrating lateral line primordium. *in situ* hybridization experiments with (L) *sall3a,* (M) *sall3b,* (N) *sall4*, and (T) *zfhx3b* were performed with the molecular crowding reagent Dextran Sulfate (see Experimental Procedures for rationale). All other *in situ* hybridization experiments in this figure were performed without this reagent. Scale bar: (A-X) 30 µm.

Figure 4. A Subset of Transcription Factor Genes Lose Expression in the Spinal Cord of Zebrafish *mib1*^{ta52b} Mutant Embryos at 24 h.

(A-Y) Lateral views of (A, C, F, H, K, M, P, R, U, W) head, (B, D, G, I, L, N, Q, S, V, X) spinal cord, and (E, J, O, T, Y) tail in (A, B, F, G, K, L, P, Q, U, V) sibling and (C-E, H-J, M-O, R-T, W-Y) *mib1*^{ta52b} mutant embryos at 24 h. Rostral, left. Dorsal, up. None of the *in situ* hybridization experiments in this figure were performed with the molecular crowding reagent Dextran Sulfate. Scale bar: (A, C, F, H, K, M, P, R, U, W) 50 µm, (B, D, G, I, L, N, Q, S, V, X) 20 µm, and (E, J, O, T, Y) 25 µm.

Figure 5. A Subset of Transcription Factor Genes Show Reduced Expression in the Spinal Cord of Zebrafish *mib1*^{ta52b} Mutant Embryos at 24 h.

Lateral views of (A, C, E, G, I, K, M, O, Q, S, U, W, Y, A', C', E', G', I', K', M', O', Q') head, and (B, D, F, H, J, L, N, P, R, T, V, X, Z, B', D', F', H', J', L', N', P', R') spinal cord in (A, B, E, F, I, J, M, N, Q, R, U, V, Y, Z, C', D', G', H', K', L', O', P') sibling and (C, D, G, H, K, L, O, P, S, T, W, X, A', B', E', F', I', J', M', N', Q', R') *mib1*^{ta52b} mutant embryos at 24 h. Rostral, left.

Dorsal, up. (Y-B') *sall4 in situ* hybridization experiments were performed with the molecular crowding reagent Dextran Sulfate (see Experimental Procedures for rationale). All other *in situ* hybridization experiments in this figure were performed without this reagent. Scale bar: (A, C, E, G, I, K, M, O, Q, S, U, W, Y, A', C', E', G', I', K', M', O', Q') 50 µm, (B, D, F, H, J, L, N, P, R, T, V, X, Z, B', D', F', H', J', L', N', P', R') 20 µm.

Figure 6. A Subset of Transcription Factor Genes Show Expanded Expression in the Spinal Cord of Zebrafish *mib1*^{ta52b} Mutant Embryos at 24 h.

Lateral views of (A, C, E, G, I, K, M, O, Q, S, U, W) head, and (B, D, F, H, J, L, N, P, R, T, V, X) spinal cord in (A, B, E, F, I, J, M, N, Q, R, U, V) sibling and (C, D, G, H, K, L, O, P, S, T, W, X) *mib1*^{ta52b} mutant embryos at 24 h. Rostral, left. Dorsal, up. None of the *in situ* hybridization experiments in this figure were performed with the molecular crowding reagent Dextran Sulfate. Scale bar: (A, C, E, G, I, K, M, O, Q, S, U, W) 50 µm, (B, D, F, H, J, L, N, P, R, T, V, X) 20 µm.

Tables

Table 1. Gene Names, ZFIN Gene Identifiers, Primer Sequences and PCR Conditions for *in situ* Hybridization RNA Riboprobe Synthesis.

Column 1 lists genes analyzed in this study. Column 2 provides the unique ZFIN identification number for each gene. Columns 3 and 4 contain the primer sequences and expected product sizes (in base pairs (bp)) respectively, used to generate templates for anti-sense RNA riboprobe synthesis from 27 h wild-type cDNA. Bold and underlined text in column 3 shows the T3 RNA Polymerase minimal promoter sequence added to the reverse primers. Column 5 indicates the annealing temperature for the primer pairs shown in each row of column 3. For further conditions for riboprobe synthesis, please see Experimental Procedures.

Table 1. Gene Names, ZFIN Identifiers, Primer Sequences and PCR Conditions for *in situ* Hybridization RNA Riboprobe Synthesis.

Gene Name	ZFIN Gene ID	Primer Sequences	PCR Product Size (bp)	Annealing Temperature (°C)
aurkb	ZDB-GENE-020419-40	Forward: CTGGATTAAACAGCCGCCATG	1082	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**CAGAAGAACAGCTATAATACAGTGGAG		
foxb1a	ZDB-GENE-990616-47	Forward: CAGACGCTGCCCACTACCTC	429	67.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GGCTCAGAGATTGCGGAGAG		
foxb1b	ZDB-GENE-990415-77	Forward: AACAACAAGCAAAACTCCGAATG	1029	63.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GGTAGCCTAAATTCATGACATGC		
her8a	ZDB-GENE-030131-2376	Forward: ACCAATGCGTCACTCAGATTTG	853	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**CCATGTGGCATAAGGAACATTGTC		
homeza	ZDB-GENE-030616-592	Forward: GGCATAGCCGCTACAAAAAG	1069	62.0
		Reverse: **AATTAACCCTCACTAAAGGGA**TCTTGCAGTGGTCCTGTCTG		
ivns1abpb	ZDB-GENE-030131-6266	Forward: ATTATGCCCGTTCTGGACTG	846	63.0
		Reverse: **AATTAACCCTCACTAAAGGGA**TAAATGGGCTCCACTCGTTC		
mybl2b	ZDB-GENE-041007-1	Forward: AAAACCCCGCTCACACAGAAG	507	66.0
		Reverse: **AATTAACCCTCACTAAAGGGA**CAGTGGAGAGATAGTTTTGGGTG		
myt1a	ZDB-GENE-030131-3885	Forward: GGCTACACCAAAAGCAGCTC	1065	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**AGCTCTAGGGCAACCTGACA		
nr2f1b	ZDB-GENE-040426-1438	Forward: AGCTAAATTTGGGCTGTCAGAC	1028	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GCCCCAAATAATCAACCTGCAG		
onecut1	ZDB-GENE-040426-1469	Forward: GTGGACACTGCTCCGCTATATC	856	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GGAACGGAGGAGTGGTTCTG		
sall1a	ZDB-GENE-020228-2	Forward: CTCTTTAACTGGCACACACACATG	1041	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GAGAAACTGGGCCCTAAAAGATC		
sall3a	ZDB-GENE-020228-4	Forward: CGGACATCCCTTCTCCAGATTC	1026	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**ATTCCTGTGCTACATCCAGAAC		
sall3b	ZDB GENE-030131-9140	Forward: CAGTGAAATCAACTCCGAGCTTG	957	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GGCTTGCTGTCAAATATGGAGAC		
sall4	ZDB GENE-060328-2	Forward: CTCCGCTCTATGACCCTCAG	1189	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GTGCAAAGTGTCTGGCGATA		
sox2	ZDB-GENE-030909-1	Forward: CCCACCTACAGCATGTCCTATTC	970	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**TCTAACAGATGAAGAGTGGGAGAC		
sox19b	ZDB-GENE-010111-1	Forward: TTAAACCAGAACCCCTGTCG	1112	62.0
		Reverse: **AATTAACCCTCACTAAAGGGA**CTGACAGCGAAGATCAGTGC		

Gene Name	ZFIN Gene ID	Primer Sequences	PCR Product Size (bp)	Annealing Temperature (ºC)
sp8b	ZDB-GENE-030131-3654	Forward: CGACGTGTAACAAAATCGGGAG	683	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**AGACGAGAGAACCGGTTTGAATC		
tsc22d1	ZDB-GENE-030131-7785	Forward: GACCTTGGAGTTTCTTTGTGCTG	347	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**TTCACCAGATCCATGGCTTGTTC		
wdhd1	ZDB-GENE-030131-1665	Forward: TTATGGTCACTCTGAAGGCCAC	748	65.0
		Reverse: **AATTAACCCTCACTAAAGGGA**ATCCCAAATCAGCAGGTTTCCTC		
zfhx3b	ZDB-GENE-030131-7577	Forward: AGCCCATCCTCATGTGTTTC	1197	63.0
		Reverse: **AATTAACCCTCACTAAAGGGA**GGTTCTGGGTGCATTCACTT		
znf804a	ZDB-GENE-070912-632	Forward: TCATGCAAGCAAGAGAGTGG	1172	63.0
		Reverse: **AATTAACCCTCACTAAAGGGA**CAGTCCTCTGCAGGGCTAAC		
znf1032	ZDB-GENE-080305-4	Forward: TATCGTGGTCCCGTAATCTACAC	366	64.0
		Reverse: **AATTAACCCTCACTAAAGGGA**TTCTCGTGTGGTGTTTAAGGTG		

Figure 1

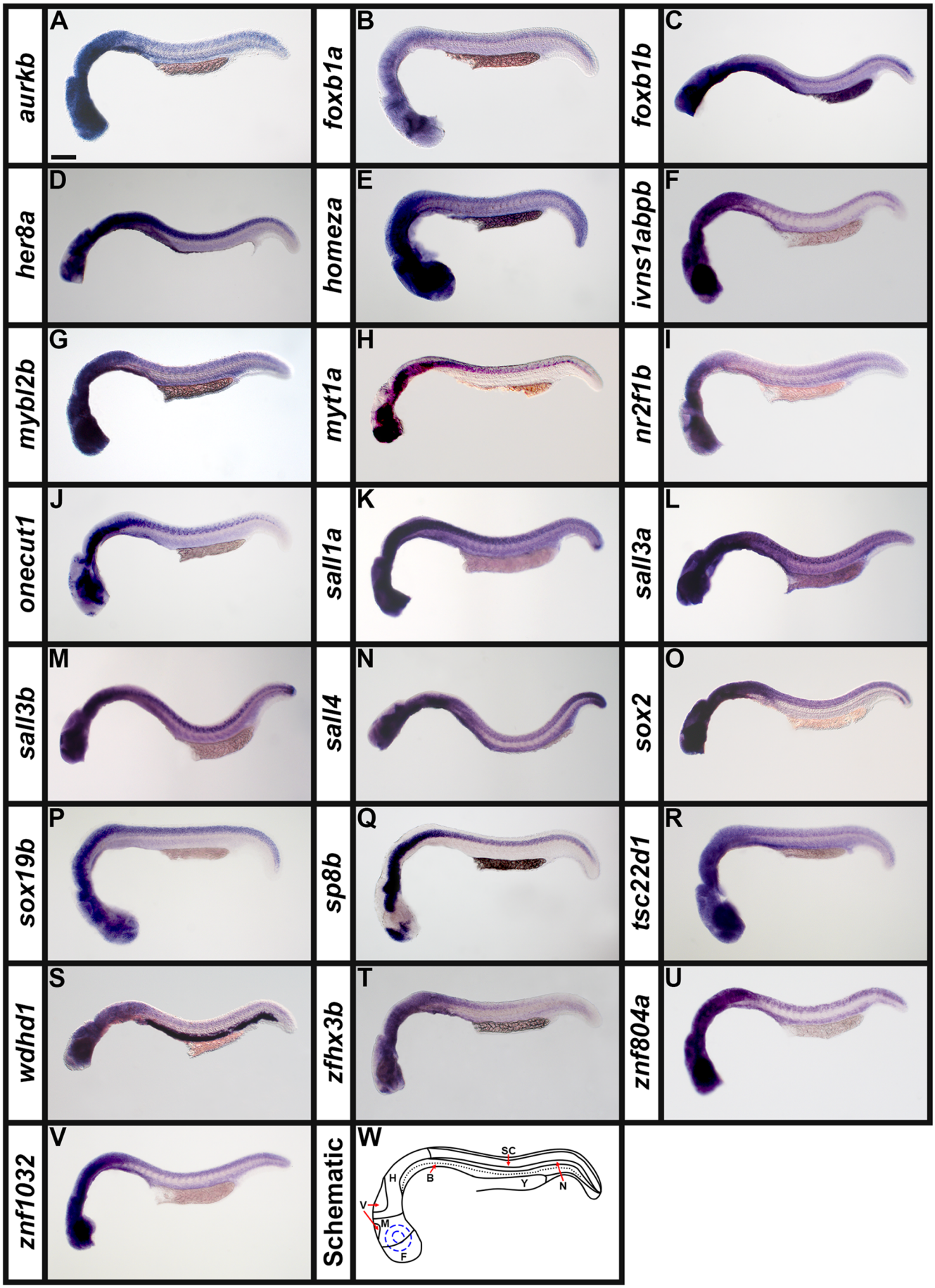
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Figure 2

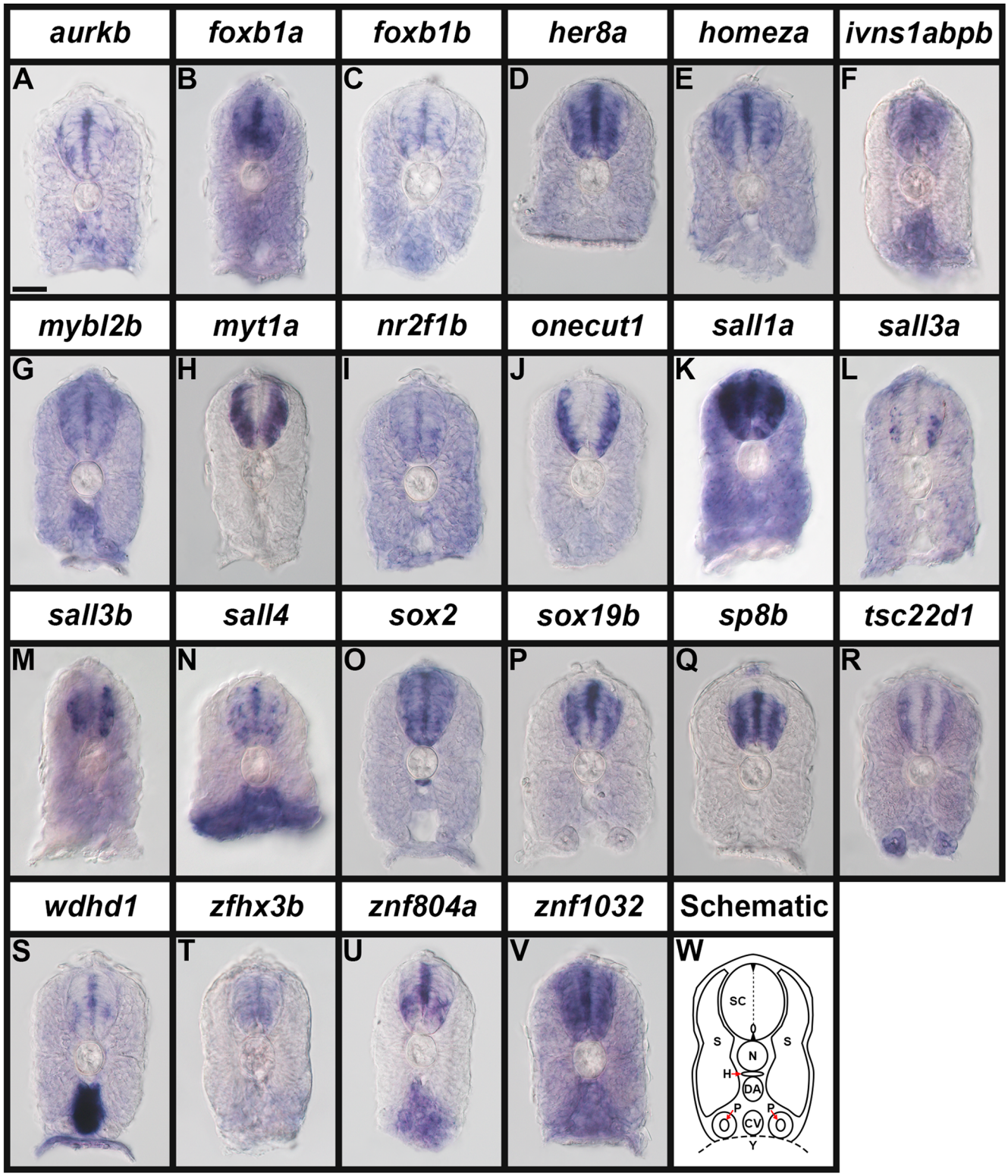
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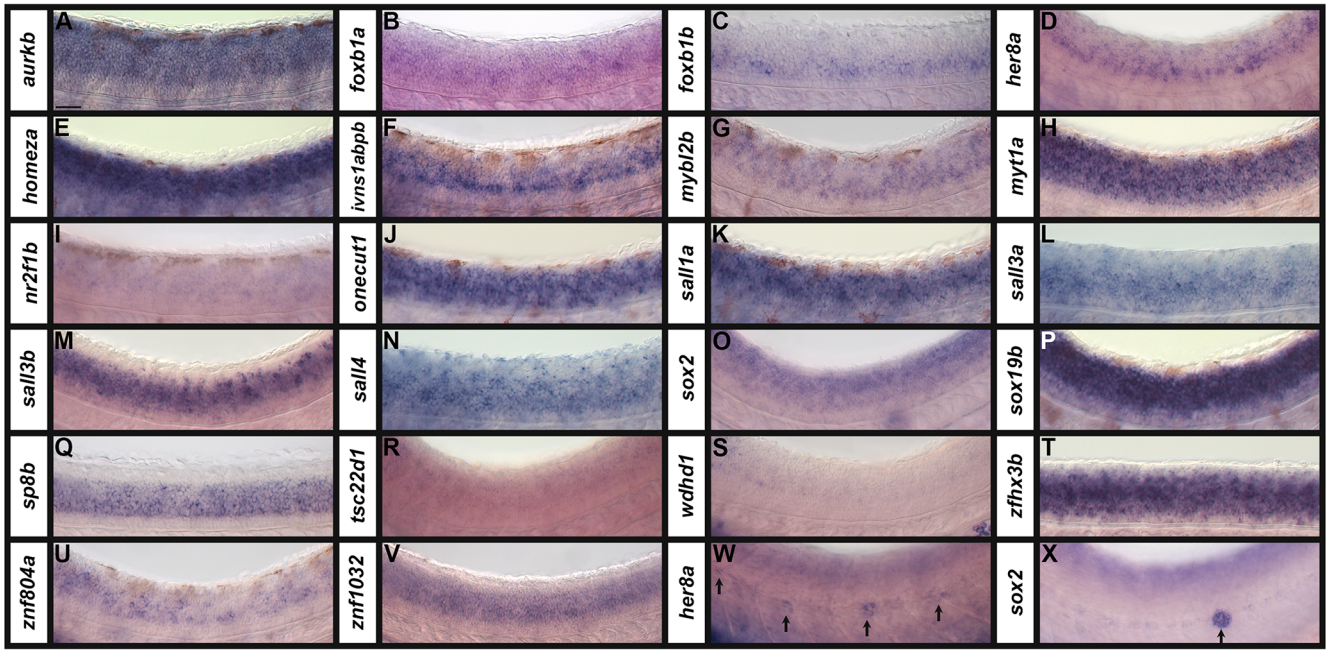
Figure 3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M
sp8b
Q
znf804a
U
foxb1a
B
ivns1abpb
F
onecut1
J
sall4
N
tsc22d1
R
znf1032
V
foxb1b
C
mybl2b
G
sall1a
K
sox2
O
wdhd1
S
her8a
W
her8a
D
myt1a
H
sall3a
L
sox19b
P
zfhx3b
T
sox2
X

Figure 4

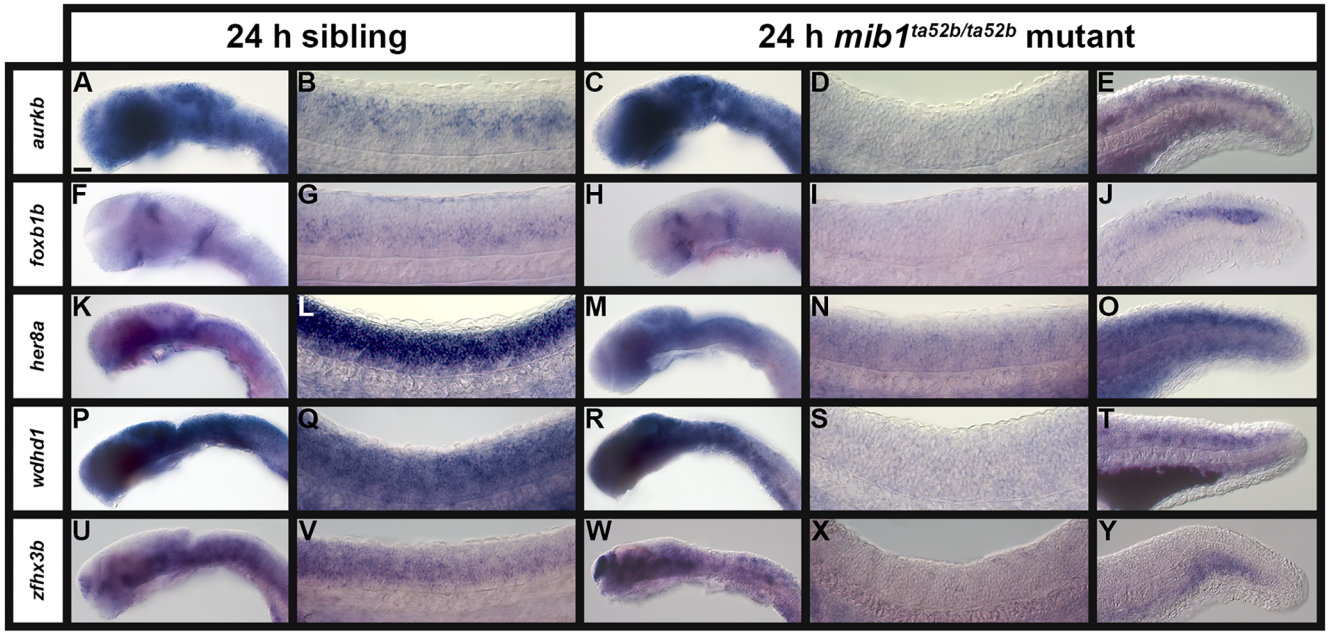

24 h sibling
24 h mib1ta52b/ta52b mutant
aurkb
foxb1b
her8a
wdhd1
zfhx3b
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y

Figure 5

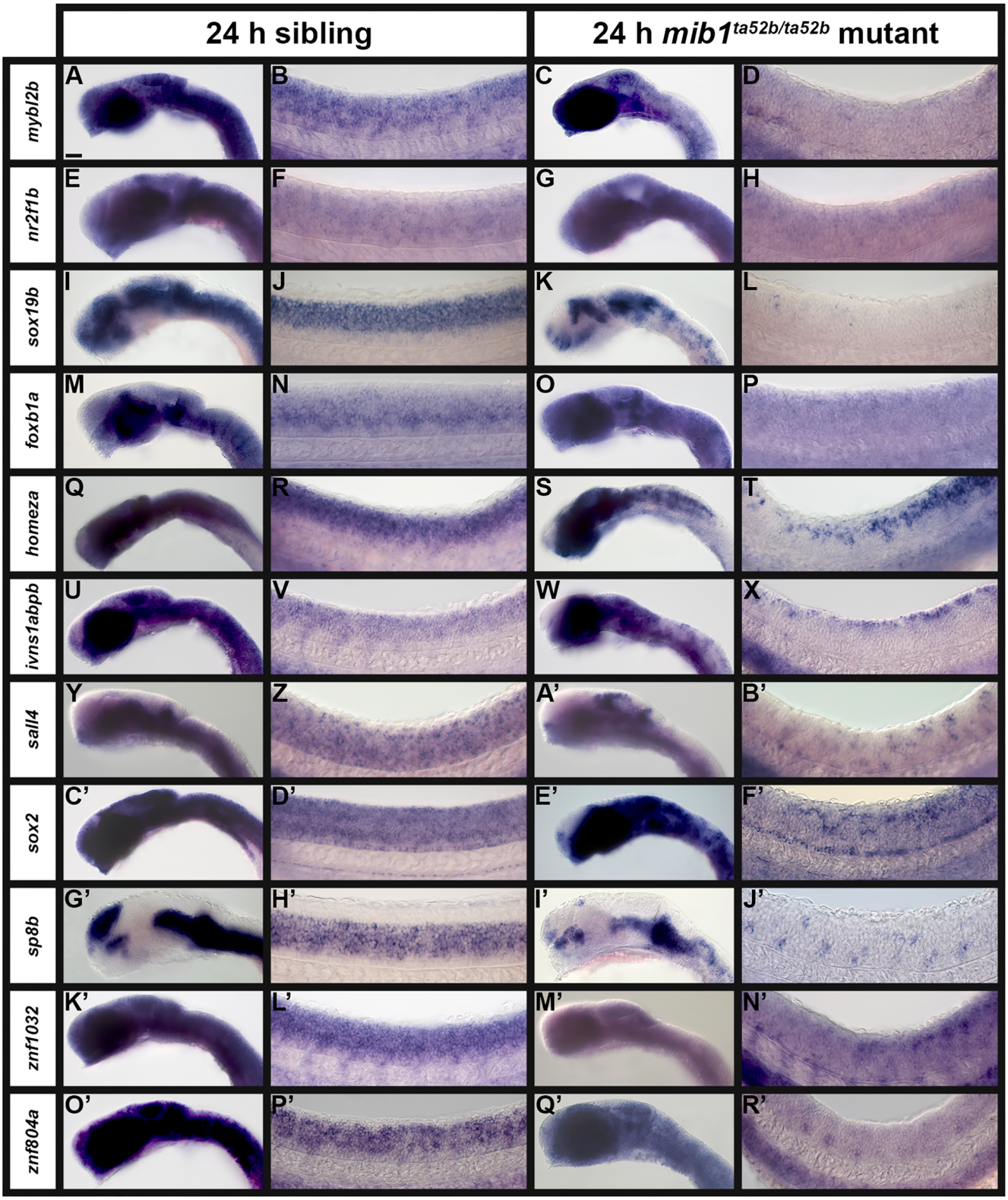
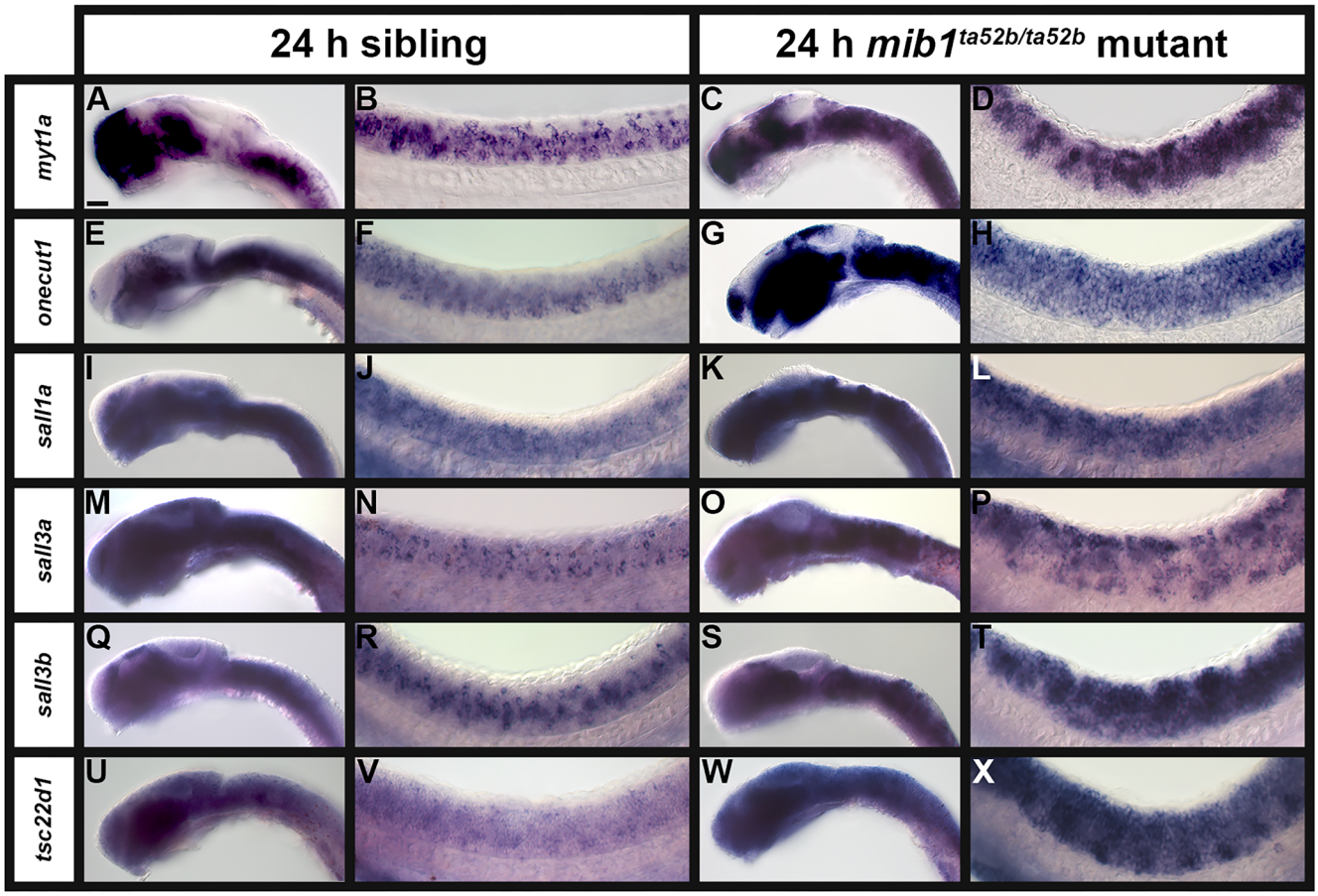

24 h sibling
24 h mib1ta52b/ta52b mutant
mybl2b
nr2f1b
sox19b
foxb1a
homeza
ivns1abpb
sall4
sox2
sp8b
znf1032
znf804a
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
A'
B'
C'
D'
E'
F'
G'
H'
I'
J'
K'
L'
M'
N'
O'
P'
Q'
R'

Figure 6


24 h sibling
24 h mib1ta52b/ta52b mutant
myt1a
onecut1
sall1a
sall3a
sall3b
tsc22d1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X