Tal1, Gata2a and Gata3 have distinct functions in the development of V2b and cerebrospinal fluid-contacting KA spinal neurons

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Key words: CSF-cN, *tal2*, *sox1*, GABA, *pkd2l1, vsx1,* V3, V2c

Abstract

Vertebrate locomotor circuitry contains distinct classes of ventral spinal cord neurons which each have particular functional properties. While we know some of the genes expressed by each of these cell types, we do not yet know how several of these neurons are specified. Here, we investigate the functions of Tal1, Gata2a and Gata3 transcription factors in the specification and development of two of these populations of neurons with important roles in locomotor circuitry: V2b neurons and cerebrospinal fluid-contacting Kolmer-Agduhr (KA) neurons (also called CSF-cNs). Our data provide the first demonstration, in any vertebrate, that Tal1 is required for correct development of KA neurons and Gata3 is required for correct development of V2b neurons. We also uncover differences in the genetic regulation of V2b cell development in zebrafish compared to mouse. In addition, we demonstrate that Sox1a and Sox1b are expressed by KA and V2b neurons in zebrafish, whereas in mouse, Sox1 is expressed by V2c neurons. KA neurons can be divided into ventral KA'' neurons and more dorsal KA' neurons. Consistent with previous morpholino experiments, our mutant data suggest that Tal1 and Gata3 are required in KA' but not KA'' cells, whereas Gata2a is required in KA'' but not

KA’ cells, even though both of these cell types co-express all three of these transcription factors. Our analyses of zebrafish single and double mutants suggest that Gata2a is required to specify KA’’ and repress V3 fates in cells that normally develop into KA’’ neurons. In *gata2a* mutants, cells in the KA’’ domain lose expression of most KA’’ genes and there is an increase in the number of cells expressing V3 genes. In contrast, our data suggest that Gata3 and Tal1 are both required for KA’ neurons to differentiate from progenitor cells. In the KA’ domain of these mutants, cells no longer express KA’ markers and there is an increase in the number of mitotically-active cells. Finally, our data demonstrate that all three of these transcription factors are required for later stages of V2b neuron differentiation and that Gata2a and Tal1 have different functions in V2b development in zebrafish than in mouse.

13 Main Figures; 3 Supplementary Data Figures; 7 Supplementary Data Tables

10714 words

Introduction

During development, neural circuits need to be precisely assembled for correct behavioral repertoires to be established. In the ventral spinal cord, several distinct classes of neurons, with particular functional properties, must be specified in correct numbers and locations and make appropriate connections with other neurons and muscle cells for locomotor circuitry to properly function. There are still many unanswered questions about how this occurs. Most studies so far, suggest that the development of distinct spinal neurons is regulated by the transcription factors that each cell type express. For example, some transcription factors control when cells differentiate, others determine the overall identity of the cell and some specify particular functional properties such as axon trajectory, neurotransmitter phenotype, and/or expression of particular neuropeptides (e.g. Moran-Rivard et al., 2001; Gross et al., 2002; Muller et al., 2002; Lanuza et al., 2004; Sapir et al., 2004; Cheng et al., 2005; Pillai et al., 2007; Batista and Lewis, 2008; Hilinski et al., 2016; Juárez-Morales et al., 2016). However, for many classes of spinal neurons, including several of those involved in locomotor circuity, we still don't know which transcription factors regulate these different aspects of specification and differentiation. In this paper we investigate the functions of Tal1, Gata2a and Gata3 transcription factors in the development of two classes of ventral spinal neurons with crucial roles in locomotor circuitry: cerebrospinal fluid-contacting Kolmer-Agduhr (KA) neurons (also called CSF-cNs), and V2b neurons.

KA neurons were identified almost a hundred years ago in over 200 vertebrates by Kolmer and Agduhr (hence the name "KA neurons"). These cells are GABAergic and have ipsilateral ascending axons. Notably, they are located near the central canal and their apical dendritic extensions extend microvilli and a motile cilium into the canal and contact cerebrospinal fluid (CSF) (e.g. Kolmer, 1921; Agduhr, 1922; Vigh et al., 1977; Barber et al., 1982; Dale et al., 1987; Bernhardt et al., 1992; Roberts et al., 1995; Stoeckel et al., 2003). This suggests that these neurons may modulate spinal cord functions in response to changes in CSF composition and/or flow. Consequently, more recently they have also been called CSF contacting neurons (CSF-cNs). CSF-cNs/KA neurons can be divided into distinct ventral and dorsal populations called KA" or KA' neurons respectively (Fig. 1; Park et al., 2004; Djenoune et al., 2014; Petracca et al., 2016). KA'' neurons are located in the most ventral part of the spinal cord and originate from the most ventral progenitor domain, the p3 domain, which also produces V3 ventral interneurons (Park et al., 2004; Schäfer et al., 2007; Djenoune et al., 2014; Petracca et al., 2016). In contrast, KA' neurons are located slightly more dorsally and in zebrafish they originate from the progenitor domain that is located just above the p3 domain, the pMN domain, which also produces

82 motoneurons (MNs) (Park et al., 2004; Djenoune et al., 2014). However, in mouse these more dorsal
83 KA' cells originate from both the pMN domain and the domain just above it, the p2 domain, which
84 also generates V2 interneurons (Petracca et al., 2016). Excitingly, recent studies have started to
85 elucidate the functions of these KA neurons/CSF-cNs and have discovered differences between them.
86 In mouse, the two classes of cells have distinct electrophysiological properties: most KA' cells have
87 repetitive spiking whereas KA'' cells only fire once (Petracca et al., 2016). In zebrafish, KA' and KA''
88 neurons express distinct neuropeptides, have slightly different axonal and dendritic morphologies and
89 have both overlapping and distinct synaptic partners and functions in locomotor circuitry (Bohm et al.,
90 2016; Hubbard et al., 2016; Djenoune et al., 2017). For example, KA' neurons respond to lateral
91 bending of the spinal cord and regulate both the duration and the frequency of slow locomotion while
92 KA'' neurons respond to longitudinal contractions and regulate posture during fast locomotion (Bohm
93 et al., 2016; Hubbard et al., 2016; Djenoune et al., 2017). However, despite these recent advances in
94 understanding KA neuronal properties and functions we currently know very little about how these two
95 cell types are specified.
96
97 V2b neurons (also called VeLDs in zebrafish) develop dorsal to KA neurons, from the p2 domain.
98 Similar to KA neurons, they are GABAergic, and their axons are ipsilateral, but in contrast to KA
99 neurons, V2b axons descend towards the caudal end of the spinal cord. V2b neurons also have
100 important functions in locomotion circuitry. For example, V2b neurons prevent extensor and flexor
101 muscles from contracting simultaneously, so enabling the alternating muscle contraction that is
102 essential for walking (Al-Mosawie et al., 2007; Batista et al., 2008; Kimura et al., 2008; Joshi et al.,
103 2009; Zhang et al., 2014; Britz et al., 2015). However, like KA neurons, we still do not fully
104 understand how the development of V2b neurons is genetically regulated.
105
106 Zebrafish KA'', KA' and V2b cells all express *tal1* (previously called *scl*), *gata3* and *gata2a*
107 (previously called gata2, (gata2b is not expressed in spinal cord, Lewis Lab unpublished data); Batista
108 et al., 2008; Kimura et al., 2008; Butko et al., 2015). *gata2a* and *gata3* encode C4 zinc-finger
109 transcription factors and *tal1* encodes a basic helix-loop-helix transcription factor. All three of these
110 transcription factors are also expressed in amniote V2b cells (Nardelli et al., 1999; Zhou et al., 2000;
111 Karunaratne et al., 2002; Smith et al., 2002; Li et al., 2005; Muroyama et al., 2005; Al-Mosawie et al.;
112 Del Barrio et al., 2007; Peng et al., 2007) and Gata2 and Gata3 are expressed by amniote CSF-cN / KA
113 neurons (Petracca et al., 2016; expression of Tal1 was not examined). In mouse, Gata2 is required for
114 generation of correct numbers of both V2a and V2b cells (Nardelli et al., 1999; Zhou et al., 2000;

Francius et al., 2015), but it is not clear whether the "missing" V2 cells die, fail to differentiate or transfate into a different cell type in these mouse *Gata2* mutants. In contrast, when Tal1 function is eliminated in the mouse CNS, V2b cells transfate into V2a cells (Muroyama et al., 2005; Joshi et al., 2009). However, in both of these mouse mutants, CSF-cNs/ KA neurons were not analyzed.

In contrast, experiments in zebrafish have examined functions of Gata2a and Gata3 in KA cells but not V2b neurons. Interestingly, morpholino knock-down of Gata2a in zebrafish resulted in a loss of GABAergic and *gata3*- and *tal2*- expressing cells in the KA'' domain (the most ventral row of the spinal cord) but not the more dorsal KA' domain, whereas morpholino knock-down of Gata3 resulted in a loss of GABAergic and *tal2*-expressing cells in the KA' but not the KA'' domain, suggesting that even though these transcription factors are expressed by both KA' and KA'' cells, they may be differentially required by these cells (Yang et al., 2010). However, similar to the mouse *Gata2* mutant analyses discussed above, these zebrafish experiments did not determine whether the "missing" cells die, transfate or just lose their GABAergic phenotypes. In addition, morpholinos can sometimes cause non-specific off-target effects (Kok et al., 2015), so it is important to confirm these phenotypes with mutant analyses.

Tal1 function(s) in KA cells and Gata3 function(s) in V2b cells have not been examined in any vertebrate. While mouse *Gata3* mutants exist (e.g. Pandolfi et al., 1995; Pai et al., 2003; Craven et al., 2004; Zhang et al., 2004; Kurek et al., 2007) spinal interneurons have not been examined in these mutants. It is also not known whether any of these three genes act redundantly in spinal neurons.

To address all of these fundamental gaps in our knowledge, we performed detailed analyses of the spinal cord phenotypes of zebrafish *tal1, gata3* and *gata2a* single and double mutants. We also examined *sox1* expression in the zebrafish spinal cord, demonstrating for the first time that *sox1a* and *sox1b* are expressed by both KA'' and KA' neurons. These *sox1* experiments also revealed that, unlike in amniotes where Sox1 is expressed by a small subset of V2 cells called V2c cells, zebrafish *sox1* genes are expressed by at least most V2b neurons, suggesting that V2c cells may not exist in zebrafish. Interestingly, our *tal1, gata3* and *gata2a* mutant analyses suggest that each of the transcription factors encoded by these genes is only required in some of the spinal neurons that co-express these genes. Gata2a is required in KA'' neurons to specify KA'' fates and repress V3 fates, but it is not required for correct development of KA' neurons. In contrast neither Gata3 nor Tal1 are required for correct development of KA'' neurons, either singly or redundantly, but both of these transcription factors are

required for KA' neurons to differentiate from progenitor cells. Finally, we also demonstrate that all three of these transcription factors are required for later stages of V2b neuron development.

Materials and Methods

Ethics approval

All zebrafish experiments in this research were carried out in accordance with the recommendations of, and were approved by, the Syracuse University IACUC committee.

Zebrafish husbandry and fish lines

Zebrafish (*Danio rerio*) were maintained on a 14-hour light / 10-hour 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), *Tg(-8.1gata1:gata1-EGFP)* (Kobayashi et al., 2001), *gata2a*^{um27} (Zhu et al., 2011), *gata3*^{sa0234} (described here) or *tal1*^{t21384} (Bussmann et al., 2007) fish. Embryos were staged in hours post fertilization at 28.5°C (h) according to (Kimmel et al., 1995).

The *Tg(-8.1gata1:gata1-EGFP)* (Kobayashi et al., 2001) transgenic line expresses EGFP in all KA (KA'' and KA') neurons and some V2b neurons (Batista et al., 2008).

The *gata2a*^{um27} and *tal1*^{t21384} mutants have been previously described and are both presumed to be null alleles (Bussmann et al., 2007; Zhu et al., 2011). The *gata2a* mutation is a 10bp deletion that creates a stop codon upstream of both zinc finger domains (Zhu et al., 2011). The *tal1* mutation is a nonsense mutation that produces a stop codon at amino acid 183, upstream of the C-terminus of the protein, including the entire bHLH domain (Bussmann et al., 2007). Therefore, in each case, even if a truncated protein is made, it should be unable to bind DNA.

The *gata3*^{sa0234} mutation was created using zinc finger nucleases by Huw Williams, Steve Harvey and Ross Kettleborough in the Stemple Lab at the Wellcome Trust Sanger Centre. If translated, this mutant allele would encode a truncated protein with 13 aberrant amino acids after the Threonine at position 264, followed by a premature stop. As a result, only 8 amino acids of the first zinc finger domain would remain intact, and the second zinc finger would be completely lost (Supplementary Figure 1), strongly suggesting that this is a null allele. Consistent with this, our analyses of KA cell expression of *tal2* and *gad67* in *gata3* mutants are consistent with earlier morpholino knock-down analyses of *gata3* function in KA cell development (Yang et al., 2010). We also observed a one-to-one correspondence between mutant phenotypes and a homozygous mutant genotype, consistent with the phenotypes resulting from the loss of Gata3 function.

Genotyping

DNA for genotyping was isolated from both anesthetized adults and fixed embryos via fin biopsy or head dissections respectively. PCR and restriction enzyme digest assays or KASP assays designed by LGC Genomics LLC, using DNA extracted from head dissections, were used to identify fish carrying mutations. KASP assays use allele-specific PCR primers, which differentially bind fluorescent dyes that we quantified with a BioRad CFX96 real-time PCR machine to distinguish genotypes. The proprietary primers used are: Gata2_um27 (*gata2a* genotyping), Gata3_sa0234 (*gata3* genotyping) and Tal1_t21384(*tal1* genotyping).

Heads of fixed embryos were dissected in 70% glycerol / 30% phosphate-buffered saline (PBS) with insect pins. Embryo trunks were stored in 70% glycerol / 30% PBS at 4°C for later analysis. DNA was extracted via the HotSHOT method (Truett et al., 2000) using 20µL of 50mM NaOH and 2µL of 1M Tris-HCl (pH-7.5).

The *gata2a*^{um27} mutation results in a 10bp deletion and was PCR genotyped using primers and protocol described in (Zhu et al., 2011). The PCR produces a 98bp product from the mutant allele and a 108bp product from the WT allele. These products were separated and identified using a 2% Super Fine Resolution (SFR) agarose gel. Alternatively, a different PCR was performed using forward 5'TTTTCCGTGACCCTGTGTTC and reverse 5'ACTCACCAGTCTGCGCTTTG primers and reaction conditions of: 98°C for 60 sec followed by 34 cycles of 94°C for 30 sec, 61°C for 45 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. This PCR reaction generates a product of 264 bp, which was digested using Msp1. Msp1 does not cut the WT PCR product, but cuts the mutant PCR product generating 164bp and 100bp fragments.

tal1^{t21384} mutants were identified by PCR using forward 5'TTTCATGCGCATATCCAAAA and reverse 5'GAAAATCCGTCGCACAACT primers and the following conditions: 98°C for 3 min followed by 34 cycles of 94°C for 30 sec, 54°C for 45 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. This PCR reaction generates a product of 180bp. This was digested using the DdeI restriction enzyme, which does not cut the 180bp WT PCR product but cuts the mutant PCR product to generate 160bp and 20bp fragments.

gata3^{sa0234} mutants were genotyped by PCR using the following primers: forward 5'GGTTGTGTAGTTGTGCTTGC and reverse 5'TTCTGTCCGTTCATCTTGTG and the following conditions: 98°C for 60 sec followed by 34 cycles of 94°C for 30 sec, 58°C for 45 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. This generates a PCR product of 240bp. This was digested using the Hinf1 restriction enzyme, which does not cut the mutant PCR product but cuts the WT PCR product to generate 159bp and 81bp fragments. These products were separated and identified using a 2.5% Super Fine Resolution (SFR) agarose gel.

in situ Hybridization and Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde and single *in situ* hybridization or fluorescent *in situ* hybridization plus immunohistochemistry experiments were performed as previously described (Concordet et al., 1996; Batista et al., 2008). Embryos older than 24h were often incubated in 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigment formation. For fluorescent *in situ* hybridization + immunohistochemistry, after detection of the *in situ* hybridization reaction using TSA Kit #5, with HRP, Goat anti-mouse IgG and Alexa Fluor 594 Tyramide (ThermoFisher Scientific, T20915), embryos were washed 8 x 15 minutes in PBST (PBS with 0.1% Tween-20) and incubated in Image-iT FX Signal Enhancer (ThermoFisher Scientific, I36933) for 30 minutes at room temperature. Immunohistochemistry was performed using the following primary antibodies: chicken polyclonal anti-GFP primary antibody (Abcam, Ab13970, 1:500), mouse anti-Nkx6.1 (F55A12, Develpmental Studies Hybridoma Bank, Iowa, 1:500), rabbit anti-phospho-Histone H3 (Ser10; Millipore #06-570; 1/500), rabbit anti-activated Caspase-3 (Fisher Scientific/BD, BDB559565, 1:500), a mixture of rat anti-Islet-1 and rat anti-Islet-2 (Developmental Studies Hybridoma Bank, Iowa antibodies 39.4D5 and 40.2D6 were mixed 1:1 and used at a final concentration of 1:300). The secondary antibodies used were: goat anti-rabbit Alexa Fluor 568 (ThermoFisher Scientific, A-11036, 1:1000), and a goat anti-chicken IgY (H+L), Alexa Fluor 488 secondary antibody (ThermoFisher Scientific, A-11039, 1:1000) and goat anti-mouse Alexa Fluor 488 (ThermoFisher Scientific, A-11029, 1:1000). Both double *in situ* hybridization and immunohistochemistry plus *in situ* hybridization double labeling experiments were performed as previously reported (Batista et al., 2008). Immunohistochemistry for GFP and pH3 was performed as described in Juárez-Morales et al. (Juárez-Morales et al., 2016) and immunohistochemistry for Islet1/2 was performed as described in Lewis and Eisen (Lewis and Eisen, 2004). Cross-sections were cut by hand using a razor blade mounted in a 12 cm blade holder (World Precision Instruments, Cat. # 14134). In cases where expression of a particular gene is lost in a specific domain, we checked for low levels of expression of that gene by substantially over-staining embryos.

To determine neurotransmitter phenotypes, we used *in situ* probes of genes that function as transporters of neurotransmitters or that synthesize specific neurotransmitters as these are some of the most specific molecular markers of these cell fates (Higashijima et al., 2004b; Higashijima et al., 2004c; and references therein). A mixture of probes to *slc17a6a* and *slc17a6b* (previously called *vglut*), which encode glutamate transporters, was used to label glutamatergic neurons (Higashijima et al., 2004b; Higashijima et al., 2004c). GABAergic neurons were labeled by a mixture of probes to *gad1b* and *gad2* genes (probes previously called *gad67a, gad67b* and *gad65*) (Higashijima et al., 2004b; Higashijima et al., 2004c). The *gad1b* and *gad2* genes encode for glutamic acid decarboxylases, which are necessary for the synthesis of GABA from glutamate. The *sox1a* and *sox1b* probes were synthesized from plasmids obtained from Dr. Uwe Strähle (Karlsruhe Institute of Technology, Germany) from the library described by Armant and colleagues (Armant et al., 2013). *gata2a*, *gata3*, *sst1.1* and *urp1* probe templates were PCR amplified from 27h WT zebrafish cDNA. The PCR primers used are provided in Supplementary Table 1. cDNA was prepared as described previously (England et al., 2017). In all cases, reverse primers contained the T3 RNA polymerase promoter binding site used to synthesize the antisense RNA probe. PCR conditions were: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 56.5°C for 30 s, 72°C for 1.5 min and a final extension step of 72°C for 10 min. All other probes were synthesized from plasmids that have previously been reported. For details and corresponding references please see Supplementary Table 2.

To assess expression of Caspase3, rabbit Anti-Activated Caspase-3 (Fisher Scientific/BD, BDB559565, 1:500) was used as described previously (Hilinski et al., 2016). 24h embryos were fixed in 4% PFA at 4^0C overnight, washed 3 times in PBST, permeabilized with acetone for 20 min at -20^0C and then washed 3 X 5 min with PBS. Embryos were then blocked (Blocking Solution: Triton 0.5%, BSA 2%, DMSO 10%, Goat Serum 2%, and PBS) for 2 hours at room temperature and incubated overnight with a Anti-Activated Caspase-3 primary antibody diluted 1:500 in blocking solution at 4^0C. The next day, embryos were washed for 8 X 15 min in PBTX (PBS with 0.5%, Triton and 5% DMSO) and incubated with goat Anti-Rabbit Alexa 488 (1:500) in PBTX +2% BSA for 4 hours at room temperature. Embryos were washed 8 X 15min in PBTX and mounted in 2% DABCO solution for imaging and analysis.

Imaging

Embryos were mounted in 70% glycerol, 30% PBS and differential interference contrast (DIC) pictures were taken using an AxioCam MRc5 camera mounted on a Zeiss Axio Imager M1 compound

microscope. A Zeiss LSM 710 confocal microscope was used to image fluorescent *in situ* and fluorescent immunohistochemistry experiments. All confocal images were processed using Image J software (Abràmoff et al., 2004), in which case appropriate numbers of focal planes were merged using maximum intensity projections. For some NBT-BCIP ISH experiments, multiple planes were merged in Image J using minimum intensity projections in order to show labeled cells at different medial lateral positions in the spinal cord. All images were processed for brightness-contrast and color balance using Adobe Photoshop software (Adobe, Inc). Figures were assembled using Adobe Illustrator (Adobe, Inc).

Counting cells

Embryos from single and double mutant crosses were counted blind to genotype. The row immediately dorsal to the notochord is denoted as row 1 and rows dorsal to this are assigned in ascending order (e.g. Fig. 1A & D). In all cases, cell counts are for both sides of a five-somite length of the spinal cord adjacent to somites 6-10. Embryos were mounted laterally with the somite boundaries on each side of the embryo exactly aligned and the apex of the somite over the middle of the notochord. This ensures that the spinal cord is straight along its dorsal-ventral axis and that cells in the same dorsal/ventral position on opposite sides of the spinal cord will be directly above and below each other. In some cases the medial-lateral location of labeled cells was also determined to distinguish between V2b and KA cells. KA cells are medial and V2b cells are more lateral. Based on our analyses of several different genes expressed by KA and/or V2b cells, we assigned medial cells in row 1 expressing these genes as KA'' cells, medial cells in rows 2 and 3 expressing these genes as KA' cells and lateral cells in rows 3 and above expressing these genes as V2b cells (e.g. Fig. 1 A-E). However, note that *gad* genes are also expressed in more dorsal spinal cells (e.g. see Batista and Lewis, 2008). Labeled cells in embryos analyzed by DIC were counted while examining embryos on a Zeiss Axio Imager M1 compound microscope. We identified somites 6-10 in each embryo and counted the number of labeled cells in that stretch of the spinal cord. We adjusted the focal plane as we examined the embryo to count cells at all medial/lateral positions (both sides of the spinal cord; also see Batista et al., 2008; Batista and Lewis, 2008; England et al., 2011; Hilinski et al., 2016; Juárez-Morales et al., 2016). Cell counts for fluorescently-labeled cells were performed by analyzing all focal planes in a confocal stack of the appropriate region of the spinal cord. For Islet1/2 positive cells, cells in the two most dorsal rows, which correspond to Rohon-Beard neurons, were not counted. Only ventral cells that correspond to motoneurons were counted. These also have smaller nuclei than the more dorsal Rohon Beard cells. For pH3-positive cells, cell rows were assigned based on average cell diameters. In all cases, values

317 are reported as the mean +/- the Standard Error of the Mean (SEM). Results were analyzed using the
318 Student's t-test. n values for each experiment are provided in the figure legends.
319

Results

Tal1 is required for expression of *gata3*, *gata2a* and *tal2* in the normal KA' spinal domain

We have previously shown that KA'', KA' and V2b spinal neurons express *tal1* (Batista et al., 2008; see also Fig. 1 B, C, E), but the function(s) of Tal1 in these cells has not been investigated. Therefore, as a first step to determining if Tal1 is required for correct development of these cells, we examined other markers of KA and V2b cells in a zebrafish *tal1* mutant (Bussmann et al., 2007).

In wild type (WT) embryos, *gata3*, *gata2a* and *tal2* are all expressed by KA'' and KA' neurons (Pinheiro et al., 2004; see also Fig. 1F, I, L; Batista et al., 2008). In addition, *gata3* and *gata2a* are expressed by V2b neurons and *tal2* is expressed by a subset of V2b neurons (Fig. 1F, I, L; Supplementary Figure 2; also see Batista et al., 2008; Yang et al., 2010). In *tal1* mutants, we found no change in the number of cells expressing any of these genes in row 1 (Fig. 1F-N and 2; Supp. Table 3). In WT embryos, row 1 cells that express these genes are KA'' neurons (Batista et al., 2008; see also Fig. 1 and materials and methods). We also found no change in the number of cells expressing *gata3* or *gata2a* in row 4 or above (Figs 1 F-K and 2; Supp. Table 3), although *tal2* expression was lost in this domain (Fig. 1 L-N and 2; Supp. Table 3). In WT embryos, the cells that express these genes in this domain are V2b neurons (Batista et al., 2008; see also Fig. 1 and materials and methods). In contrast, there was a dramatic reduction in the number of cells expressing each of these three genes in rows 2 and 3 in *tal1* mutants (Figs 1F-N and 2; Supp. Table 3). In WT embryos, the row 2 cells that express these genes are KA' cells but row 3 cells that express these genes might be KA' or V2b cells (Batista et al., 2008; see also Fig. 1A-D and materials and methods). KA' and V2b cells can be distinguished by their locations and soma-morphologies. KA' cells are medial and have more circular soma, whereas V2b cells are lateral and have pear-shaped soma (Fig. 1A-C). All of the remaining row 3 *gata3*- and *gata2a*-expressing cells in the *tal1* mutants were pear shaped and located laterally, consistent with them being V2b cells (Fig. 1C; Supp. Table 4). The most likely explanation of these results is that either KA' cells are lost in *tal1* mutants or that *tal1* is required for expression of *gata3*, *gata2a* and *tal2* in KA' cells. In contrast, these data suggest that *tal1* is not required for expression of *gata3*, *gata2a* or *tal2* in KA'' cells or *gata3* or *gata2a* in V2b cells, although it is required for expression of *tal2* in V2b cells.

Gata3 and Gata2a are required for expression of *gata* and *tal* genes in KA' and KA" spinal domains respectively

Previous morpholino experiments suggested that Gata3 and Gata2a may have distinct functions in KA' and KA'' cells respectively (Yang et al., 2010). However, morpholinos can cause non-specific defects (Kok et al., 2015) and the function of these genes in zebrafish V2b neurons was unknown. Therefore, to determine if Gata3 and/or Gata2a are required for correct development of zebrafish V2b or KA cells, we examined markers of these cell types in a *gata2a* mutant (Zhu et al., 2011) and a newly-generated *gata3* mutant (see materials and methods).

In *gata3* mutants, we found no change in the number of cells expressing *gata2a*, *tal1* or *tal2* in row 1 (KA'' domain; Figs 3 B-J and 2; Supp. Table 3). However, there was a complete loss of expression of all three genes in row 2 and a statistically significant reduction in the number of cells expressing each gene in row 3 (KA' domain; Figs 3 B-J and 2; Supp. Table 3). As with *tal1* mutants, all of the remaining labeled row 3 cells were located laterally, suggesting that none of these cells are KA' neurons (Fig. 3 D, G, J; Supp. Table 5). However, in contrast to *tal1* mutants, in *gata3* mutants there was also a statistically significant decrease in the number of cells expressing *gata2a* and *tal1* in row 4 and above, suggesting that there may also be a reduction in the number of V2b cells expressing these genes (Figs 3 B-G and 2; Supp. Table 3). Together, these results suggest that *gata3* is not required for expression of *gata2a, tal1* or *tal2* in KA'' cells or most V2b cells. However, it is probably required for KA' cell differentiation, KA' cell survival or expression of *gata2a*, *tal2* and *tal1* in KA' cells.

In contrast to both *gata3* and *tal1* mutants, in *gata2a* mutants there was no statistically significant change in the number of cells expressing *gata3, tal1* or *tal2* in rows 2 or 3 (KA' domain; Figs 3 K-S and 2; Supp. Table 3). However, we very rarely observed cells expressing *gata3* or *tal1* in row 1 (KA'' domain; Figs 3 K-P and 2; Supp. Table 3). The number of cells expressing *tal2* in row 1 was unchanged, although the expression of *tal2* might be slightly reduced compared to WT embryos (Figs 3 Q-S and 2; Supp. Table 3). There was no statistically significant change in the number of cells expressing *tal1* or *tal2* in row 4 and above. However, there was a small, but statistically significant, decrease in the number of cells expressing *gata3* in this domain (V2b domain; Fig. 3M and 2; Supp. Table 3). Taken together, these data suggest that *gata2a* is not required for expression of *gata3*, *tal1* or *tal2* in KA' cells. However, our data suggest that *gata2a* is required for correct specification and/or development of KA'' cells and a small number of V2b cells. We do not think that either of these cell types are dying in *gata2a* mutants as we see normal numbers of *tal2*-expressing cells in row 1 and normal numbers of *tal1*- and *tal2*-expressing cells in row 4 and above.

sox1a/b are expressed by KA'', KA' and V2b neurons in zebrafish

In all of the cases described above where there is a phenotype in the V2b domain, there is only a partial reduction in the number of cells expressing the gene in question. In mice, a small subset of V2b cells becomes a distinct population of cells called V2c cells (Panayi et al., 2010). Therefore, we hypothesized that the affected cells in our mutants might be V2c cells. However, it had not yet been established if V2c cells exist in zebrafish. In mouse, V2c cells are the only V2 cells that express Sox1 (Panayi et al., 2010). Therefore, we examined spinal cord expression of *sox1a* and *sox1b,* the zebrafish orthologs of mouse *Sox1* (Okuda et al., 2006). Interestingly, we found that both *sox1a* and *sox1b* are expressed in similar numbers of cells not just in the V2 spinal domain but also in the KA'' and KA' domains (Fig. 4), suggesting that these two genes might be co-expressed by both V2 and KA neurons. Co-localization experiments between *sox1a*, *sox1b* and *gata3* confirmed that *sox1a* and *sox1b* are co-expressed in all KA'' and KA' neurons and, unlike in mouse, both of these *sox1* genes are expressed in at least most, and probably all, V2b neurons (Fig. 4). We do observe occasional cells that express just one of these genes, but this probably reflects slight differences in the timing of gene expression and/or low levels of expression that are hard to detect in these double labeling experiments.

Tal1, Gata3 and Gata2a are required for normal expression of *sox1a/b* in KA and V2b domains

As *sox1a* and *sox1b* are expressed in V2b, KA'' and KA' cells we examined the expression of these genes in *tal1*, *gata3* and *gata2a* mutants. In *tal1* mutants, there was no effect on the number of row 1 cells expressing *sox1a* or *sox1b*, but there was a dramatic reduction in the number of cells expressing these genes in rows 2 and 3 (KA' domain; Figs 5 B-G and 2; Supp. Tables 3 and 4). Interestingly there was also a severe reduction in the number of row 4 and above cells expressing *sox1b* but only a slight reduction in the number of these cells expressing *sox1a* (V2b domain; Figs 5 B-G and 2; Supp. Table 3). In *gata3* mutants, there was also no effect on the number of row 1 cells expressing *sox1a* or *sox1b*, but there was a complete loss of *sox1a* and *sox1b* expression in rows 2 and 3 (KA' domain; Figs 5 H-M and 2; Supp. Tables 3 and 5). There was no effect on the number of row 4 and above cells expressing *sox1a* but there was a slight reduction in the number of these cells expressing *sox1b* (V2b domain; Figs 5 H-M and 2; Supp. Table 3). In contrast, in *gata2a* mutants there was no change in the number of cells expressing *sox1b,* but there was a reduction in the number of cells expressing *sox1a* in rows 1 and 2 (Figs 5 N-S and 2; Supp. Table 3).

Taken together, our results so far reveal that in *gata3* and *tal1* mutants, expression of all of the genes that we have examined is lost in the KA' domain (row 2 and medial row 3 cells), whereas in *gata2a*

mutants expression of most of the genes that we have examined is lost in the KA'' domain (row 1). There are several possible explanations for these phenotypes. We think it unlikely that KA or V2b cells are mis-localized in any of these mutants, as we didn't observe an increase in the number of cells expressing any of these genes in any spinal cord domains. Our data also suggest that cells that would normally develop as KA'' neurons are not dying in *gata2a* mutants as normal numbers of *sox1b*-and *tal2*-expressing cells are present in row 1 (Fig. 3Q-S and Fig 5Q-S). However, KA'' neurons could be mis-specified as a different cell type or just not expressing *sox1a*, *gata3* and *tal1*. In *gata3* and *tal1* mutants, KA' neurons could be dying, mis-specified as a different cell type or just not expressing the genes that we have examined.

KA' cells do not die or transfate into motoneurons or V2a cells in *tal1* or *gata3* mutants

To test whether KA and/or V2 neurons were dying in any of these mutants we used an activated caspase-3 antibody, as in previous zebrafish studies (Sorrells et al., 2013; Hilinski et al., 2016). However, we did not detect any increase in the number of labeled cells in *tal1*, *gata3* or *gata2a* mutants compared to their WT siblings (Fig. 6 A-F).

To investigate whether KA or V2b neurons might be mis-specified as a different cell type in these mutants, we examined markers of motoneurons and V2a neurons, as these cell types are also present in KA and V2 domains in WT embryos at these stages. Motoneurons express Islet1/2 (Park et al., 2004) and V2a cells express *vsx1* (Kimura et al., 2006; Batista et al., 2008; Kimura et al., 2008). However, there was no change in the number of cells expressing either of these markers in any of the three mutants (Fig. 6 G-R; Supp. Table 6). This suggests that no cells in these mutants are mis-specified as motoneurons or V2a neurons.

Tal1 and Gata3 are required for *gad* and neuropeptide expression in KA' domain and Gata2a is required for *gad* and neuropeptide expression in KA'' domain. All three proteins are required for *gad* expression in some V2b cells.

As the precise functions of Tal1, Gata3 and Gata2a in KA and V2b development were still unclear, we next examined later functional properties of these cells, such as neurotransmitter and neuropeptide phenotypes, axonal projections and presence of a crucial channel protein. Our aims for these experiments were two-fold. First, we wanted to establish if any of the cells that have lost *sox1a, sox1b, gata2a*, *gata3*, *tal1* and/or *tal2* expression still develop into functional KA or V2b neurons with appropriate morphological and/or physiological characteristics. Second, as the genes that we had

examined so far are all expressed relatively early during KA and V2b development, we wanted to test if any of the cell types that had normal expression of these genes developed phenotypes later in development.

KA'', KA' and V2b neurons are all GABAergic (Bernhardt et al., 1992; Batista et al., 2008). Therefore, we examined expression of *gad1b* and *gad2* (referred to here as *gad*), which are specifically expressed by GABAergic cells (see materials and methods; *gad1b* and *gad2* encode for glutamic acid decarboxylases, which are necessary for the synthesis of GABA from glutamate). We found that in both *tal1* and *gata3* mutants, *gad* was expressed by normal numbers of cells in row 1 (KA'' domain), but almost no cells in rows 2 and 3 (KA' domain; Figs 7 A-F and 2; Supp. Tables 3 - 5). There was also a reduction in the number of GABAergic cells in row 4 and above (V2b domain), although this reduction was much more pronounced in *tal1* mutants than in *gata3* mutants (Figs 7 A-F and 2; Supp. Table 3). In contrast, there were almost no *gad*-expressing row 1 cells in *gata2a* mutants (KA'' domain) and there was also a statistically significant reduction in the number of *gad*-expressing cells in row 2 and row 4 and above (KA' and V2b domains) (Figs 7 G-I and 2; Supp. Table 3). In agreement with these single mutant data, almost all *gad*-expressing cells were lost in the spinal cords of *tal1;gata2a* double mutants (Supp. Fig. 3).

KA" neurons express *urp1* (Quan et al., 2015) and KA' neurons express *sst1.1* neuropeptides (Djenoune et al., 2017). We found that *sst1.1* expression was lost in both *tal1* and *gata3* mutants (Figs 7 J-M; 2) but there were normal numbers of *sst1.1*-expressing KA' neurons in *gata2a* mutants (data not shown). In contrast, *urp1* expression was lost in *gata2a* mutants (Fig. 7 N-O) but there were normal numbers of *urp1*-expressing KA" cells in *gata3* and *tal1* mutants (data not shown).

Taken together, these data suggest that KA'' neurons with appropriate neurotransmitter and neuropeptide phenotypes are not found in *gata2a* mutants, KA' neurons with appropriate neurotransmitter and neuropeptide phenotypes are not found in either *tal1* or *gata3* mutants and fewer than normal GABAergic V2b neurons are found in all three of these mutants. However, these data also suggests that KA' neurons develop normally in *gata2a* mutants and KA'' neurons develop normally in *tal1* and *gata3* mutants.

Tal1 and Gata3 are required for *pkd2l1* expression in KA' domain and Gata2a is required for *pkd2l1* expression in KA'' domain

The non-selective cation-channel gene *pkd2l1* is expressed in both populations of KA neurons in all vertebrates examined so far (Djenoune et al., 2014; Petracca et al., 2016; England et al., 2017) and experiments in zebrafish suggest that Pkd2l1 is crucial for correct KA neuronal function in locomotor circuitry (Bohm et al., 2016). We also find *pkd2l1* expression in occasional V2b neurons (Fig. 8A; England et al., 2017). In *tal1* mutants, *pkd2l1* was expressed in normal numbers of cells in row 1 but its expression was lost in the rest of the spinal cord (Figs 8 B, E and 2; Supp. Table 3). Similarly, in *gata3* mutants, *pkd2l1* was expressed in normal numbers of row 1 cells but was not expressed in row 2. However, in contrast to *tal1* mutants, the number of *pkd2l1*-expressing cells in row 4 and above in *gata3* mutants was similar to WT embryos (Figs 8 C, F and 2; Supp. Table 3). Finally, in *gata2a* mutants, there was no change in the number of *pkd2l1*-expressing cells in rows 2 or 3, but expression of *pkd2l1* was almost eliminated in row 1 and there was a slight reduction in the number of *pkd2l1*-expressing cells in row 4 and above (Figs 8 D, G and 2; Supp. Table 3). In agreement with these single mutant data, all *pkd2l1*-expressing cells were lost in the spinal cords of *tal1;gata2a* double mutants (Supp. Fig. 3). As with the neurotransmitter and neuropeptide data discussed above, these results suggest that KA'' neurons with appropriate functional properties are not found in *gata2a* mutants and KA' neurons with appropriate functional properties are not found in either *tal1* or *gata3* mutants. These data also suggests that KA' neurons develop normally in *gata2a* mutants and KA'' neurons develop normally in *tal1* and *gata3* mutants.

KA" neurons have normal ipsilateral ascending axonal projections in *tal1* and *gata3* mutants and KA' neurons have normal ipsilateral ascending axonal projections in *gata2a* mutants.

To investigate if *tal1*, *gata3* or *gata2a* are required for correct axonal morphology of KA and/or V2b neurons we generated mutant fish transgenic for *Tg(-8.1gata1:gata1-EGFP)*, which labels all KA and some V2b neurons (Batista et al., 2008). In *tal1* mutants, KA" neurons express GFP and have normal WT-like axonal projections that are ipsilateral and ascend towards the brain (Fig. 9B). However, there is no expression of GFP more dorsally in the spinal cord, in either the KA' or V2b domains (Fig. 9B). In *gata3* mutants, both V2b and KA" neurons express GFP and appear to have WT-like axonal projections. The KA'' axons are ipsilateral and ascending and the V2b axons extend ventrally to the midline and then descend ipsilaterally (Fig. 9C). However, there are no GFP-labeled KA' cells (Fig. 9C). In *gata2a* mutants, both KA' and V2b neurons express GFP and have WT-like axonal projections but only a very small number of GFP-positive KA" neurons remain, although these also have WT-like axonal projections (Fig. 9D; it is also possible that these are KA' neurons that have moved ventrally in

the absence of KA'' neurons). These results, like the data discussed above, suggest that KA' neurons develop normally in *gata2a* mutants and KA'' neurons develop normally in *tal1* and *gata3* mutants

Do *tal1*, *gata3* and/or *gata2a* act redundantly in KA and/or V2b development?

Taken together, our results so far suggest that in *tal1* and *gata3* mutants KA'' neurons develop normally and in *gata2a* mutants KA' neurons develop normally. While we cannot rule out that there are phenotypes that we have not detected, all of the genes that we have examined are expressed normally in the cells in question, including both early-expressed transcription factor genes and later-expressed genes that encode functional properties of these neurons. In addition, the axons of these neurons follow their normal trajectories. Our analyses also suggest that some V2b neurons develop normally in *gata2a* and *gata3* mutants. However, it was possible that *tal1* and *gata3* might act redundantly in KA'' neurons and/or that *gata2a* and *gata3* might act redundantly in V2b neurons. Therefore, we examined these cells in the respective double mutants.

To test if *tal1* and *gata3* act redundantly in KA'' neuronal development we examined functional properties of KA'' neurons in *tal1*;*gata3* double mutants. Using *Tg(-8.1gata1:gata1-EGFP)* we found that, just like in single mutants, KA" neurons have normal ipsilateral ascending axonal projections in *tal1*;*gata3* double mutants (Fig. 9E). In addition, KA'' neurons are still GABAergic and express *pkd2l1* normally in *tal1*;*gata3* double mutants (*gad*-expressing and *pkd2l1*-expressing cells are present in normal numbers in the KA'' domain; Fig. 10 C, E, G, H; Supp. Table 7). This suggests that *tal1* and *gata3* are not required, even redundantly, for the correct development of KA" neurons.

To test whether *gata2a* and *gata3* act redundantly in V2b cells we examined *gata2a*;*gata3* double mutants. Using *Tg(-8.1gata1:gata1-EGFP)* we could not detect any GFP-expressing V2b cells (Fig. 9F; Supp. Table 7) in these double mutants. In addition, there were considerably fewer *gad*-expressing cells in the V2b domain of *gata2a*;*gata3* double mutants than there were in either single mutant (Fig. 10 B, D). Unlike the *Tg(-8.1gata1:gata1-EGFP)* experiment, some *gad*-expressing cells remain in double mutants, but these are likely be more dorsal GABAergic spinal neurons such as V1 cells or dI4 or dI6 cells (Batista and Lewis, 2008; Hilinski et al., 2016; Juárez-Morales et al., 2016). These results suggest that *gata2a* and *gata3* are required either additively or redundantly in V2b neurons.

In *gata2a* mutants KA'' cells may transfate into V3 neurons

Our data suggest that KA'' cells do not die in *gata2a* mutants, as there is no increase in the expression of a cell death marker in these mutants (Fig. 6F) and there is no change in the number of row 1 cells expressing *sox1b* or *tal2* (Figs 3 Q-S, 5 Q-S and 2). However, KA'' cells do not develop correctly in these mutants as they lose expression of all of the other KA'' genes that we have examined and they do not develop correct KA'' functional characteristics. As these cells are no longer GABAergic, we tested whether they become glutamatergic (excitatory) and we found a statistically significant increase in the number of cells expressing the glutamatergic markers *slc17a6a/b* (*slc17a6a* and *slc17a6b* encode glutamate transporters, see materials and methods; Fig. 11 G-I; Supp. Table 6). While WT embryos at 24h had no glutamatergic cells in row 1, *gata2a* mutants had approximately 11 glutamatergic cells in this row in the spinal cord region adjacent to somites 6-10 (Fig.11 L; Supp. Table 6).

KA'' cells arise from the same progenitor domain (p3) as glutamatergic V3 interneurons (Park et al., 2004). V3 neurons, like KA'' cells, express *tal2* (Schäfer et al., 2007; Yang et al., 2010). However they also express a unique V3 marker *sim1a* (Schäfer et al., 2007; Yang et al., 2010). In zebrafish, most V3 neurons form later than KA neurons. Earlier reports suggested that *sim1a* is not expressed until 36h (Schäfer et al., 2007; Yang et al., 2010). However, we detected a few scattered *sim1a*-expressing cells in row 1 in 24h WT embryos (Fig. 11 N, O) and the number of these cells was statistically significantly increased in *gata2a* mutants (Fig. 11 N-P), suggesting that at least some KA'' cells are transfating into V3 neurons or acquiring a hybrid V3/KA'' identity.

To establish whether V3 neurons also express *sox1b* we performed double-labeling experiments for *sox1b* and *slc17a6a/b* at 32 hpf when V3 cells are present in larger numbers. We found several cells in row 1 that co-express these two genes, suggesting that V3 cells do indeed express *sox1b* (Fig. 11 M).

We had already established that KA'' neurons form normally in *tal1* and *gata3* single and double mutants. However, it was still theoretically possible that Tal1 and/or Gata3 might act partially redundantly with Gata2a to repress the V3 fate in KA'' cells. To test this, we examined the number of V3 cells (glutamatergic cells in row 1) in both *gata2a;tal1* and *gata2a;gata3* double mutants. However, there was no statistically significant change in the number of V3 cells in either double mutant, compared to *gata2a* single mutants (Fig. 11 J-L).

KA' cells may fail to differentiate in *gata3* and *tal1* mutants

KA' cells do not appear to be dying or transfating into neighboring cell types in *gata3* or *tal1* mutants, but they also do not express any of the normal KA' markers that we have examined. Therefore, we tested if these cells might be failing to differentiate, by quantifying mitotically-active cells with phospho-histone H3 (pH3) staining. As expected, we found no statistically significant difference in the number of pH3-positive nuclei in *gata2a* mutants compared to WT embryos (Fig. 12 A-C). However, there was an increase in the number of pH3-positive nuclei in both *tal1* and *gata3* mutants. The number of pH3-positive cells in row 1 was unchanged, consistent with normal differentiation of KA" neurons in these mutants but the number of pH3-positive cells was increased in rows 2 and 3 (the KA' and pMN domain; Fig. 12 D-I), suggesting that KA' cells may be failing to exit the cell cycle. Double labeling experiments with pH3 and *olig2* (Park et al., 2002) further confirmed these results. Both *gata3* and *tal1* mutants have a statistically significant increase in the number of pH3-positive, *olig2*-positive cells compared to WT embryos (Fig. 13 A-J; Supp. Table 6), demonstrating that there is an increase in the number of mitotically-active cells in the pMN domain, from which KA' cells are usually generated. *gata3* mutants also have a small, but statistically significant, increase in the number of pH3-positive, *olig2*-negative cells compared to WT embryos (Fig. 13 A-E; Supp. Table 6), which is consistent with the small, but statistically significant, increase in the number of pH3-positive cells in row 4 and above in these mutants (Fig. 12 F; Supp. Table 6). Our double labeling experiments with Nkx6.1 suggest that these "extra" mitotically-active cells that are not within the pMN domain, are instead within the p2 domain, as *gata3* mutants had a statistically significant increase in the number of pH3-positive, Nkx6.1-positive cells compared to WT embryos but there was no difference in the number of pH3-positive, Nkx6.1-negative cells between mutants and WTs (Fig. 13 K-O; Supp. Table 6).

Discussion

To understand how correct neural circuits form during development we need to determine how the distinct neurons that constitute these circuits are specified. In this paper, we used mutant zebrafish to test the functions of three transcription factors, Tal1, Gata2a and Gata3, in multiple aspects of KA'', KA' and V2b spinal neuron specification and development. These include correct expression of transcription factor genes; cell viability; differentiation from progenitor cells and development of essential functional characteristics, such as GABAergic neurotransmitter phenotypes, appropriate axonal trajectories, expression of appropriate neuropeptide genes and expression of a crucial channel gene *pkd2l1*. Our data suggest that Gata2a is required to specify a KA'' and repress a V3 fate in cells that would normally develop into KA'' neurons and Gata3 and Tal1 are required for KA' neurons to differentiate from progenitor cells. In addition, all three of these transcription factors are required for later stages of V2b neuron differentiation.

Gata2a is required for specification of KA'' neurons

In *gata2a* mutants, there is no longer any expression of KA'' markers *gata3*, *tal1*, *sox1a*, *GAD*, *pkd2l1* or *urp1* in the most ventral row of the spinal cord (row 1), which is where KA'' cells normally form (Figs 2, 3 K-P, 5 N-P, 7 G-I and 8D and G). In addition, only very occasional cells in this row express *Tg(-8.1gata1:gata1-EGFP)* (Fig. 9D) and it is possible that these may be KA' neurons that have moved ventrally in the absence of KA'' neurons. However, normal numbers of cells still express both *sox1b* and *tal2* in the KA'' domain of these mutants (Fig. 3 Q-S and 5 Q-S), suggesting that cells that would normally have developed into KA'' cells are still differentiating in normal numbers and are still expressing both of these genes. Consistent with this, we don't observe any increase in cell death in this domain or any increase in the number of mitotically-active cells (Figs 6F and 12A-C). *tal2* is also expressed by glutamatergic V3 cells, which usually form later than KA'' cells in row 1 (Schäfer et al., 2007; Yang et al., 2010). Our data suggest that V3 cells also express *sox1b* (Fig. 11 M). This suggests that the row 1 *tal2*- and *sox1b*-expressing cells in *gata2a* mutants might be ectopic V3 cells. Consistent with this, we found an increase in the number of glutamatergic cells in row 1 and the number of cells expressing the V3 specific gene *sim1a* in these mutants (Fig. 11 L and P). Taken together, these data suggest that Gata2a is required for the specification of KA'' neurons, and that in *gata2a* mutants, cells that would have become KA'' neurons acquire at least some V3 characteristics. Gata2a may actively repress the V3 fate in these cells, or, alternatively, it is possible that the V3 fate is a default fate for row 1 neurons. Gata2 is also expressed by amniote CSF-cN / KA neurons (Petracca et al., 2016), but its function in these cells has not yet been examined. Unlike in zebrafish, where KA'' neurons are some

of the earliest neurons to form in the spinal cord, in mouse CSF-cN / KA neurons form after most other spinal cord neurons (Petracca et al., 2016). Therefore, in future work, it would be interesting to test whether Gata2 function in ventral CSF-cN / KA'' neurons is conserved between mouse and zebrafish.

Gata3 and Tal1 are required for differentiation of KA' neurons

In *gata3* and *tal1* mutants, there are almost no row 2 or medial row 3 cells that express KA' genes *gata2a, gata3, tal1, tal2, sox1a, sox1b, GAD, pkd2l1* or *sst1.1* and there are no *Tg(-8.1gata1:gata1-EGFP)*-positive KA' neurons, suggesting that KA' neurons do not form in these mutants (Figs 1-3, 5-9). Occasional row 2 cells that express one of these genes may be slightly more dorsally-located KA'' neurons. In theory, it is possible that cells expressing KA' markers are not found in the normal KA' domain because the location of KA' neurons has changed in these mutants. However, our data does not support this hypothesis, as there is no corresponding increase in the number of cells expressing any of these genes in any other spinal cord domain. In addition, we do not think that KA' cells are dying or transfating into motoneurons or KA'', V2a or V2b neurons in these mutants, as there is no increase in the number of cells expressing cell death markers or markers of these neighboring cell types (Figs 1-3, 5-9). Instead, our data suggest that the most likely explanation for the loss of KA' neurons is that these cells are not differentiating, as there is an increase in the number of mitotically-active precursor cells in the pMN domain, from which KA' cells are normally generated, in both *gata3* and *tal1* mutants (Figs 12 and 13). Gata3 is also expressed by mouse CSF-cN / KA neurons (Petracca et al., 2016), but it is not yet known if this is also the case for Tal1 and the function of Gata3 in these cells has not yet been examined in mouse. Therefore, in future work, it would be interesting to test if the functions of these transcription factors in dorsal CSF-cN / KA' neurons are conserved between mouse and zebrafish.

gata2a, *gata3* and *tal1* are all required for normal development of at least some V2b neurons

gata2a, *gata3* and *tal1* mutants all have distinct phenotypes in spinal cord row 4 and above, which corresponds to the normal V2b domain. In each mutant, several V2b genes are expressed by a reduced number of cells in this domain, although in each case at least one V2b gene is expressed in a normal number of cells (Figs 1-3, 5, 7-9). Our data suggest that the V2b cells with a phenotype are not dying, or acquiring a hybrid or new identity as there is no increase in the number of cells expressing a cell death marker, or markers of motoneurons or KA, V2a, V1 or V0v neurons (we see no increase in the number of cells expressing KA/V2b genes, Islet1/2, *vsx1*, *gad* or *slc17a6a/b*, other than the increased number of *slc17a6a/b*-expressing cells in row 1 discussed above; Figs 1-3, 5-8). Our data also suggest that V2b cells are not mis-located in any of these mutants (for example present in more ventral KA

domains) as there is no increase in the number of cells expressing V2b genes in any other spinal cord domains. Finally, there is no change in the number of mitotically-active cells in the V2b domain in *tal1* or *gata2a* mutants (Fig. 12). We did detect a small, but statistically significant, increase in the number of mitotically-active cells in the V2b domain in *gata3* mutants (Fig. 12F). However, if *gata3* is required for some V2b cells to differentiate from late progenitor cells, similar to its function in KA' cells, it is surprising that normal numbers of cells expressing *tal2* and *sox1a* are present in the V2b domain (Figs 2, 3J and 5J). In addition, given that V2a and V2b cells are usually generated as sister cells from the same progenitor cell (Del Barrio et al., 2007; Peng et al., 2007; Batista et al., 2008; Kimura et al., 2008; Joshi et al., 2009), if some V2b neurons are failing to differentiate, it is surprising that we do not also see a reduction in the number of V2a cells in these mutants (Fig. 6 O-P). Given that we also observed an slight increase, that was not statistically significant, in the number of mitotically-active cells in the V2b domain in *tal1* mutants (Fig. 12 I), an alternative explanation might be that there is an increase in the number of mitotically-active cells in the V2b domain, because a small number of the "extra" KA' progenitor cells have been displaced dorsally in these mutants.

tal1 mutants have the most severe V2b phenotype, with a complete loss of *Tg(-8.1gata1:gata1-EGFP)*-positive V2b neurons and a severe reduction in the number of GABAergic cells in row 4 and above, suggesting that at least most V2b neurons require Tal1 to develop correct functional characteristics (Figs 9B, 7B and 10E). It is possible that there are no GABAergic V2b cells in *tal1* mutants and that the small number of remaining GABAergic cells in row 4 and above are V1 neurons, a subset of which are GABAergic or more dorsal GABAergic cells (Batista and Lewis, 2008). In addition, in the V2b domain of *tal1* mutants there are no cells expressing *tal2*, almost no cells expressing *sox1b* and a reduced number of cells expressing *sox1a* (Figs 1N, 2 and 5D and G). In contrast, there are normal numbers of cells expressing *gata2a* and *gata3* in this domain, which is consistent with V2b cells at least starting to differentiate and not dying.

The *gata2a* and *gata3* V2b mutant phenotypes are less severe. However, *gata2a;gata3* double mutants have a much more severe V2b phenotype than either *gata2a* or *gata3* single mutants, suggesting that these genes either have partially-redundant functions in this cell type or they are each required in a different subset of V2b cells. Like *tal1* single mutants, *gata2a;gata3* double mutants lose expression of *Tg(-8.1gata1:gata1-EGFP)* in the V2b domain (Fig. 9F) and there is a more severe reduction in the number of row 4 and above GABAergic cells in these double mutants than in either single mutant (Fig. 10 D). As with *tal1* mutants, it is likely that at least most of the remaining dorsal GABAergic cells in

gata2a;gata3 double mutants are V1 or more dorsal cells, suggesting that there may be no GABAergic V2b cells in these double mutants.

Taken together, our data strongly suggest that *gata2a*, *gata3* and *tal1* are not required for V2b neuron differentiation or survival, but that they are instead required for later aspects of V2b neuronal development including the acquisition of correct functional characteristics.

Differences between V2b cell development in zebrafish and mouse

Interestingly our analyses have uncovered some differences in zebrafish V2b development compared to mouse. In zebrafish, *sox1a* and *sox1b* are expressed by at least most, and probably all, V2b neurons (Fig. 4), whereas in mouse, *Sox1* is only expressed by a small subset of V2b cells that turn off expression of Gata3 and develop into V2c cells (Panayi et al., 2010). This suggests that V2c cells may have evolved in tetrapods, perhaps as part of the evolution of different types of locomotion associated with limb-based movement on land (see also similar argument for subsets of V1 cells; Higashijima et al., 2004a; Lewis, 2006).

In addition, while mouse *Gata2* mutants lose all V2 cells (Nardelli et al., 1999; Zhou et al., 2000; Francius et al., 2015), in zebrafish *gata2a* mutants, we found no change in the number of cells expressing the V2a gene *vsx1* (Fig. 6 Q-R), or in the number of V2b cells expressing *tal1*, *tal2*, *sox1a* or *sox1b* (Fig. 3 N-S and Fig. 5 N-S). There was just a slight reduction in the number of V2b cells expressing *gata3, pkd2l1* or *gad* (Fig. 3 K-M, Fig. 7 G-I and Fig. 8 D & G). This suggests that Gata2 has a different function in V2 neurons in zebrafish compared to mouse, acting later in development and only in V2b cells.

Finally, while expression of several genes, and all of the functional characteristics that we analyzed, were lost in V2b cells in zebrafish *tal1* mutants, unlike in mouse, there was no change in the number of V2b cells expressing *gata2a* and there was no increase in the number of *vsx1*-expressing V2a cells (Fig. 1 I-K and Fig. 6 M-N). This suggests that Tal1 function in V2b neurons also differs between zebrafish and mouse and like with Gata2a, in zebrafish, Tal1 is probably required at a later stage of V2b development, after V2a and V2b neurons have been specified from their common progenitor cell (V2a and V2b neurons are sister cells, generated by the final division of p2 progenitor cells; Del Barrio et al., 2007; Peng et al., 2007; Batista et al., 2008; Kimura et al., 2008; Joshi et al., 2009). These differences between mouse and zebrafish are consistent with the idea that while some transcription

factor functions are highly conserved between zebrafish and mammalian spinal cord (e.g. Cheesman et al., 2004; Lewis et al., 2005; Batista and Lewis, 2008), there is also some evolutionary plasticity in the functions of other transcription factors in spinal neural development (for other examples see Hutchinson and Eisen, 2006; Hutchinson et al., 2007; Juárez-Morales et al., 2016). In future studies it would be interesting to further investigate these evolutionary differences and determine where in the vertebrate lineage they arose.

Tal1 and Gata3 are probably not required for development of KA'' neurons and Gata2a is probably not required for development of KA' neurons.

Surprisingly, even though *tal1*, *gata2a* and *gata3a* are all expressed by KA'', KA' and V2b neurons, our experiments have not revealed any requirement for *tal1* or *gata3*, either singly or redundantly, in KA'' development or for *gata2a* in KA' development. The only exception is a very slight reduction in the number of GABAergic and *sox1a*-expressing cells in row 2 in *gata2a* mutants, which could potentially be due to a few KA'' neurons being counted in row 2 in WT embryos in these experiments. In addition, while at least some V2b neurons do not develop normally in each of these three mutants, V2b phenotypes are different in each mutant (Figs 1-3, 5, 7-9), suggesting that these genes may also have distinct functions in this cell type. While we cannot rule out the possibility that cells that appear to develop normally in our experiments have phenotypes in aspects of KA and V2b neuronal development that we have not assayed, such as synapse formation or dendritic morphology, these results demonstrate that *gata2a*, *gata3* and *tal1* each have unique functions in spinal cord development. It is particularly surprising that *gata2a* and *gata3* have distinct functions in KA'' and KA' neurons as these genes encode highly-related transcription factors and act redundantly in many tissues (Haugas et al., 2016; Home et al., 2017). It is possible that even though each of these three genes is expressed in V2b, KA' and KA'' cells, each gene is only translated in a subset of these cell types. This could be tested in future studies by developing specific antibodies that work in zebrafish spinal cord, for each of these proteins. A more likely scenario might be that different co-factor proteins interact differentially with these transcription factors in each cell type.

Most of our mutant data is consistent with earlier morpholino studies

While the functions of Gata2a and Gata3 in KA neuron development had been analyzed previously using morpholino knock-down experiments (Yang et al., 2010), it was important to test these findings using mutants as morpholinos can have non-specific, off-target effects (Kok et al., 2015). Our *gata2a* and *gata3* mutant KA cell phenotypes are mainly consistent with these earlier morpholino knock-down

experiments (Yang et al., 2010). However, unlike in *gata2a* morphants, *tal2* expression is not lost in KA'' cells in *gata2a* mutants, although it may be slightly reduced (Fig. 3 Q-S). There are at last three possible explanations for this discrepancy. First, the *gata2a* morpholino may have caused a non-specific effect in KA'' cells, resulting in levels of *tal2* expression too low to detect. Second, if *tal2* expression is indeed weaker in these cells in the absence of Gata2a, it is possible that these earlier experiments did not detect its expression. Third, it is theoretically possible that the *gata2a* mutant may not be a null allele, and hence may not remove as much Gata2a activity as the morpholino. This last hypothesis seems unlikely given that the mutant lacks both zinc fingers (Zhu et al., 2011) and all of the other aspects of the phenotype are similar between the two experiments. In addition, Yang and colleagues reported that *gad* expression in the V2b domain was not affected in *gata3* knock-down embryos (Yang et al., 2010), whereas in *gata3* mutants we discovered a slight reduction in the number of V2b cells expressing *gad*. However, this reduction could have easily been missed in the earlier study, as they did not count the number of cells expressing *gad*.

Tal2 may also be required for correct KA'' cell development

Interestingly, Yang and colleagues also analyzed morpholino knock-down of *tal2* (Yang et al., 2010). These experiments suggested that Tal2 is required for KA'', but not KA' cells to become GABAergic, although expression of *gata2a* and *gata3* was unaffected in the KA'' cells. While this is a more subtle phenotype than any of our mutant phenotypes, it is striking that these data suggest that *tal2* is required specifically in KA'' but not KA' cells, which is the opposite of *tal1*, in the same way that *gata2a* is required specifically in KA'' but not KA' cells, which is the opposite of *gata3*. Along with the similarity of the *tal1* and *gata3* mutant phenotypes in KA' cells, this suggests that distinct pairs of Gata and Tal proteins may be required either as part of a complex, or in parallel, for correct development of KA'' and KA' cells. This could be tested in future work by creating *tal2* single and double mutants. There is a precedence for the idea that Tal and Gata proteins may function in a protein complex, as in mouse V2 cells, Tal1 and Gata2 form a protein complex with LMO4 and NLI (Joshi et al., 2009).

Conclusions

Taken together, the data in this paper provide substantial new insights into the spinal cord functions of *tal1*, *gata2a* and *gata3* and significantly enhance our understanding of how KA (CSF-cN) and V2b specification and differentiation are genetically regulated. Our analyses confirm many aspects of the KA phenotypes of previous morpholino knock-downs of Gata2a and Gata3. We also identify, for the first time in any vertebrate, a crucial function for Tal1 in KA' neuron differentiation and Gata3 in V2b

neuron development. Finally, we show that while Gata2a and Tal1 are also required for correct V2b neuron development in zebrafish, these transcription factors have different, later functions in these cells in zebrafish than they do in mouse.

Author Contributions

LA and SB performed most of the experiments for this paper and prepared most of the figures and tables, KK performed initial analyses of *tal1* and *gata3* mutants, SJE performed additional experiments requested by reviewers and helped to prepare figures and tables including these data, CV performed some of the genotyping of mutants and expression analyses, KL directed the study and wrote the paper with help from the other authors. All authors read and commented on drafts of the paper and approved the final version.

Funding

Funding for this project was provided by the Human Frontier Science Program Grant RGP063 (KEL), and National Institutes for Health (NIH) NINDS R21NS073979 (KEL). Research in the Lewis Lab is also supported by NIH NINDS R01 NS077947 (KEL) and National Science Foundation (NSF) IOS 1257583 (KEL). KK and CV also both received support from NSF Louis Stokes Alliance for Minority Participation grants HRD 0703452 (KK) and HRD 1202480 (CV) to Syracuse University.

Acknowledgements

We would like to thank Steve Harvey, Huw Williams and Ross Kettleborough for the *gata3* mutant, Ginny Grieb, Jessica Bouchard, Henry Putz and several SU undergraduate fish husbandry workers for help with maintaining zebrafish lines and Ginny Grieb, Leslie Vogt, William Haws, Annika Swanson, Paul Campbell and Dr. William Hilinski for help with *in situ* hybridizations and genotyping. We would also like to thank Stefan Schulte-Merker and Steve Wilson for the *tal1* mutant line, Nathan Lawson for the *gata2a* mutant line and Dr. Uwe Strähle, Sepand Rastegar and Olivier Armant for *sox1a* and *sox1b* plasmids.

Competing Interests

The authors have no competing interests.

Figure Legends

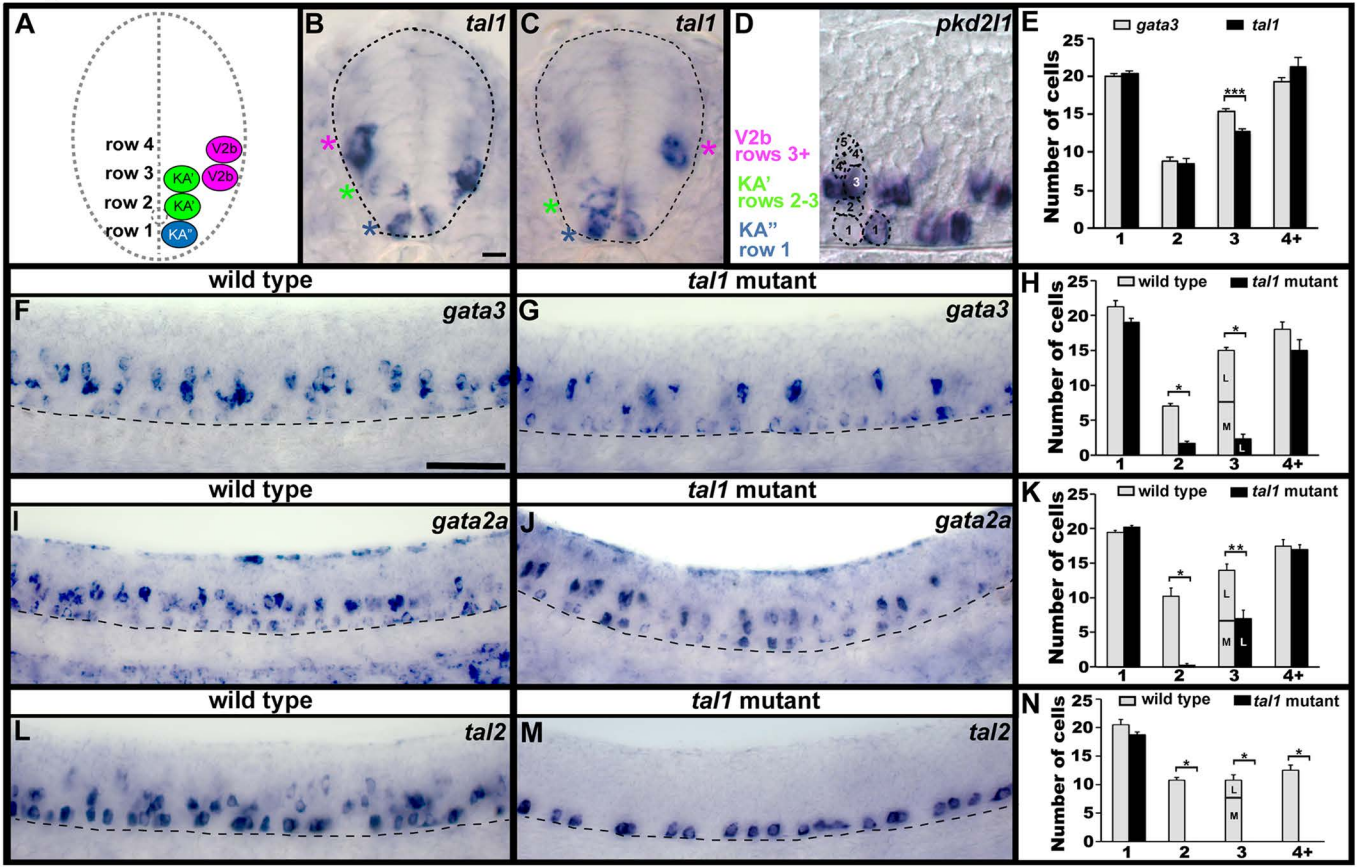
Figure 1. Spinal expression of *tal1* and requirement in KA and V2b neurons.

Cross-sectional (A-C) and lateral (D- F-G, I-J, L-M) views of 24h zebrafish embryos. Dorsal, top; in lateral views, anterior, left. (A) Schematic indicating positions of KA", KA' and V2b neurons. (B-C) *tal1* expression in KA" (blue asterisks), KA' (green asterisks) and V2b (magenta asterisks) cells. (D) Example of counting cells in different dorsal/ventral (D/V) "rows" (see materials and methods). Row 3 contains both medial KA' cells and lateral V2b cells. V2b cells are also located in row 4 and above. (E, H, K, N) Mean number of cells expressing specific genes in each D/V row of precisely-defined spinal cord region adjacent to somites 6-10. The approximate proportions of medial and lateral row 3 cells are indicated by horizontal lines separating the number of medially-located cells (bottom and indicated with an "M") from the number of laterally-located cells (top and indicated with a "L"). All of the remaining *gata3*- and *gata2a*-expressing cells in row 3 of *tal1* mutants were located laterally and were pear shaped, consistent with them being V2b cells, suggesting that no KA' cells express these genes in *tal1* mutants. *tal1* and *gata3* expression in 24h WT embryos (E). *gata3* (F-H), *gata2a* (I-K) and *tal2* (L-N) expression in WT siblings and *tal1* mutants. Dashed lines indicate spinal cord boundary (A-C) or ventral limit of spinal cord (F-M). *gata2a* expression ventral to spinal cord and in dorsal trunk is excluded from cell counts (I). Scale bars (B) = 10 microns (B-D); (F) = 50 microns (F-M). All counts were conducted blind to genotype and are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant ($p < 0.05$) comparisons are indicated with brackets and stars. *** indicates P<0.001; ** indicates P<0.01. P values are provided in Supp. Table 3.

Figure 2. Gene expression phenotypes in *gata2a*, *gata3* and *tal1* mutants

Summary schematic representation of gene expression phenotypes in *gata2a*, *gata3* and *tal1* mutants. Phenotypes are reported for cells in different cell type domains. The KA'' domain (blue) corresponds to row 1 cells, the KA' domain (green) corresponds to all row 2 plus medial row 3 cells and the V2b domain corresponds to lateral row 3 cells plus all row 4 and above cells. White boxes indicate that no cells in that domain express the gene indicated at top of column, in the mutant indicated in the most left hand column. A colored box indicates that there is no phenotype for that gene in that domain. A partially-colored box indicates a partial reduction in the number of cells expressing the gene. The amount of the box that is white is approximately proportional to the degree of reduction. Information on additional genes that are not expressed in all cell types is provided in the final column.

Figure 1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row 2
row 1
V2b
KA'
V2b
KA'
KA"
B
tal1
C
tal1
D
pkd2l1
V2b
rows 3+
KA'
rows 2-3
KA"
row 1
E
gata3
tal1
Number of cells
***
wild type
tal1 mutant
F
gata3
G
gata3
H
wild type
tal1 mutant
Number of cells
L
M
L
wild type
tal1 mutant
I
gata2a
J
gata2a
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tal1 mutant
Number of cells
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Figure 2

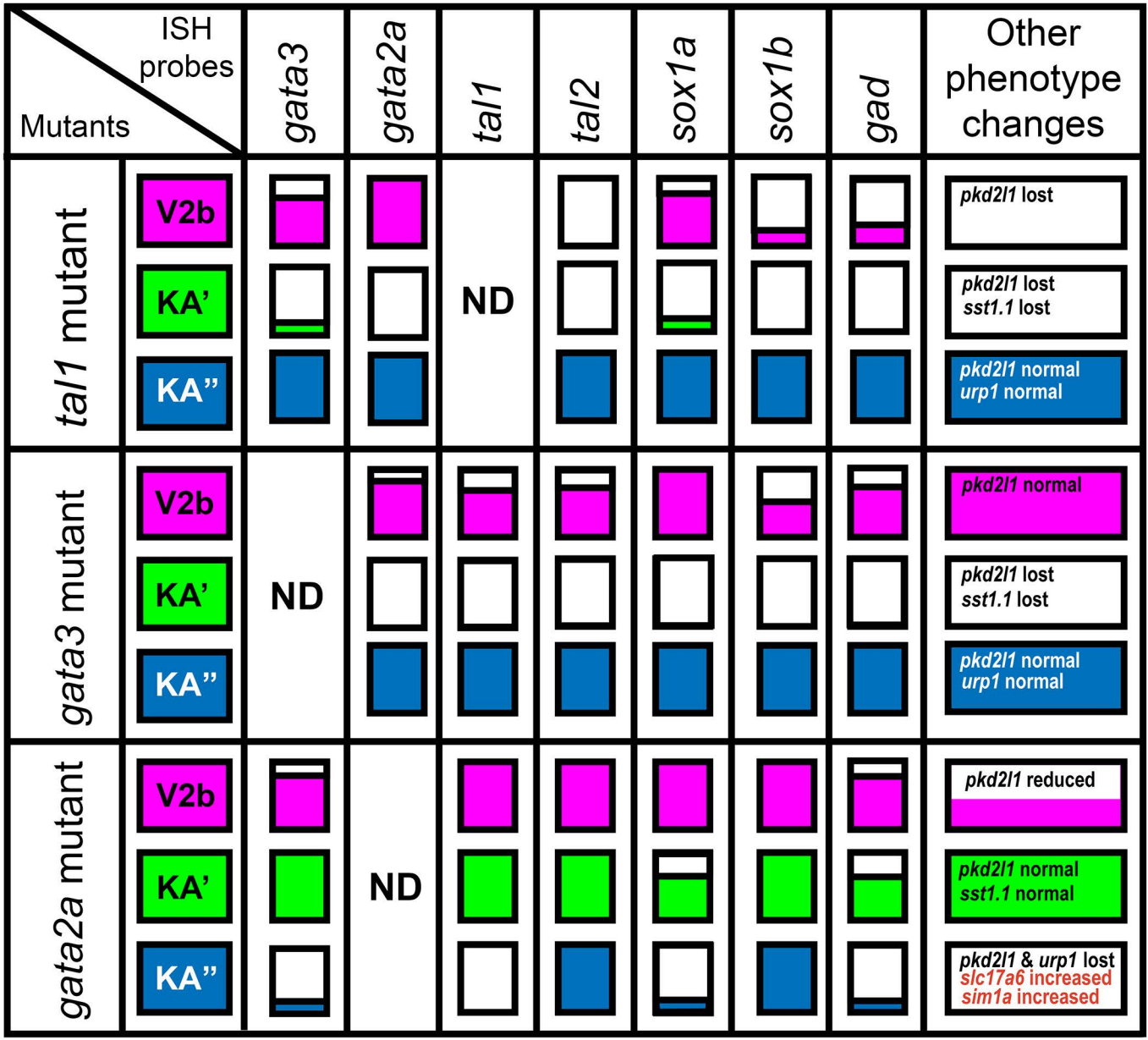

ISH probes
Mutants
gata3
gata2a
tal1
tal2
sox1a
sox1b
gad
Other phenotype changes
tal1 mutant
V2b
KA'
KA"
ND
pkd2l1 lost
pkd2l1 lost
sst1.1 lost
pkd2l1 normal
urp1 normal
gata3 mutant
V2b
KA'
KA"
ND
pkd2l1 normal
pkd2l1 lost
sst1.1 lost
pkd2l1 normal
urp1 normal
gata2a mutant
V2b
KA'
KA"
ND
pkd2l1 reduced
pkd2l1 normal
sst1.1 normal
pkd2l1 & urp1 lost
slc17a6 increased
sim1a increased

Figure 3. *gata2a*, *gata3*, *tal1* and *tal2* expression in KA", KA' and V2b neurons is differentially regulated by *gata3* and *gata2*a.

(A) Cross sectional schematic indicating positions of KA", KA' and V2b neurons. Lateral views of *gata2a* (B, C), *tal1* (E, F, N, O), *tal2* (H, I, Q, R) and *gata3* (K, L) expression in 24h WT siblings (B, E, H, J, N, Q) and *gata3* (C, F, I) and *gata2a* (L, O, R) mutants. Dorsal, top; in lateral views, anterior, left. White asterisk in E indicates blood expression of *tal1*. Dashed lines indicate ventral limit of spinal cord. Scale bar=50 microns. Mean number of cells expressing each gene in each D/V row of spinal cord region adjacent to somites 6-10 (D, G, J, M, P, S). For *gata3* mutant results (D, G, J) the approximate proportions of medial and lateral row 3 cells are indicated by horizontal lines separating the number of medially-located cells (bottom and indicated with an "M") from the number of laterally-located cells (top and indicated with a "L"). All of the remaining *gata2a*- and *tal1*-expressing cells in row 3 of *gata3* mutants were located laterally, suggesting that they are V2b cells and that no KA' cells express these genes in *gata3* mutants. More dorsal (row 4 and above) cells expressing *tal2* in *gata3* mutants (C) are more weakly stained than the strongly stained KA'' cells and are in a different focal plane. All counts are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. *** indicates P<0.001; **= indicates P<0.01; * indicates P<0.05. For P values see Supp. Table 3.

Figure 4. *sox1a* and *sox1b* are co-expressed in KA", KA' and V2b cells.

Cross-sectional (A-C) and lateral (F-H) views of 24h WT zebrafish embryos. Dorsal, top; in lateral views, anterior is left. Dashed lines indicate spinal cord boundary (A-C) or ventral limit of the spinal cord (F-H). (A) Schematic indicating positions of KA", KA' and V2b neurons. (B) *sox1a* and (C) *sox1b* expression in KA" (blue asterisks), KA' (green asterisks) and V2b (magenta asterisk) cells. (D) Mean number of cells expressing *gata3*, *sox1a* or *sox1b* in each D/V row of spinal cord region adjacent to somites 6-10. All counts are an average of at least 5 embryos. Error bars indicate SEM. Statistically significant (P<0.05) comparisons are indicated with square brackets and stars. *** indicates P<0.001; ** indicates P<0.01. There are no statistically significant differences between the numbers of cells expressing each of these genes in rows 1 and 2. While there are differences in rows 3 and 4, these are small and may reflect slight differences in timing or levels of expression and/or "noise" between different experiments as each gene was analyzed in different embryos. For P values of all comparisons see Supp. Table 3. (E) Quantification of single and double-labeled cells from double fluorescent *in situ* hybridization experiments (F-H). Each column indicates the mean number of cells +/- SEM expressing each gene and co-expressing the two genes. Double fluorescent *in situ* hybridization co-expression

experiments showing expression of *sox1a* (green) and *sox1b (red)* (F), *sox1a* (green) and *gata3* (red) (G), *sox1b* (green) and *gata3* (red) (H). Bottom row panels are single confocal plane magnified views of the dashed box regions in panels above. White asterisks in F6, G6 and H6 indicate cells co-expressing both genes whereas white crosses indicate single-labeled cells. Dorsal labeling in F1-H3 is background accumulated from several confocal planes. As labeling is often weaker in double *in situ* hybridization experiments, cells that are apparently single-labeled may co-express the other gene at levels undetected in these experiments. Consistent with this, slightly fewer cells are labeled with each *in situ* hybridization probe in the double-labeling experiments than in the single *in situ* hybridization experiments. In addition, occasional single-labeled cells may result from one of the genes being expressed slightly earlier than the other. Scale bars (B) = 10 microns (B, C); (F1) = 50 microns (F1-H3).

Figure 5. Expression of *sox1a* and *sox1b* in *tal1*, *gata3* and *gata2a* mutants.

(A) Cross sectional schematic indicating positions of KA", KA' and V2b neurons. Lateral views of *sox1a* and *sox1b* expression in 24h WT sibling (B, E, H, K, N, Q) and *tal1* (C, F), g*ata3* (I, L) and *gata2a* (O, R) mutants. Dorsal, top; anterior, left. Dashed lines indicate ventral limit of spinal cord. Scale bar = 50 microns. Mean number of cells expressing *sox1a* (D, J, P) or *sox1b* (G, M, S) in each D/V row of spinal cord region adjacent to somites 6-10 in WT and mutant embryos. For *tal1* and *gata3* mutant results (D, G, J, M) the approximate proportions of medial and lateral row 3 cells are indicated by horizontal lines separating the number of medially-located cells (bottom and indicated with an "M") from the number of laterally-located cells (top and indicated with a "L"). All of the remaining row 3 cells in these mutants are lateral, consistent with them being V2b cells. All counts are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. *** indicates $P<0.001$; * indicates $P<0.05$. For P values see Supp. Table 3.

Figure 6. Activated caspase3 and Islet1/2 immunohistochemistry and expression of *vsx1* in wild type and mutant embryos.

Lateral views of 24h WT sibling and mutant embryos as indicated. Dashed lines mark ventral limit of spinal cord. Dorsal, top; anterior, left. Scale bar = 50 microns. (A-F) Immunohistochemistry for activated Caspase-3. Very few labeled cells are seen in WT or mutant embryos (0-3 cells in a 5 somite-length of spinal cord). (G-L) Immunohistochemistry for Islet-1 and Islet-2. The smaller labeled nuclei in the ventral spinal cord correspond to motoneurons. The larger more dorsal nuclei belong to Rohon-Beard cells. There were no statistically significant differences in the number of Islet1/2 expressing

motoneurons between WT and mutant embryos (see Supp. Table 6 for average numbers of cells counted and P value for the comparison). (M-R) *vsx1* expression in V2a neurons. There were no statistically significant differences between the number of V2a neurons in WT and mutant embryos (see Supp. Table 6 for average numbers of cells counted and P value for the comparison).

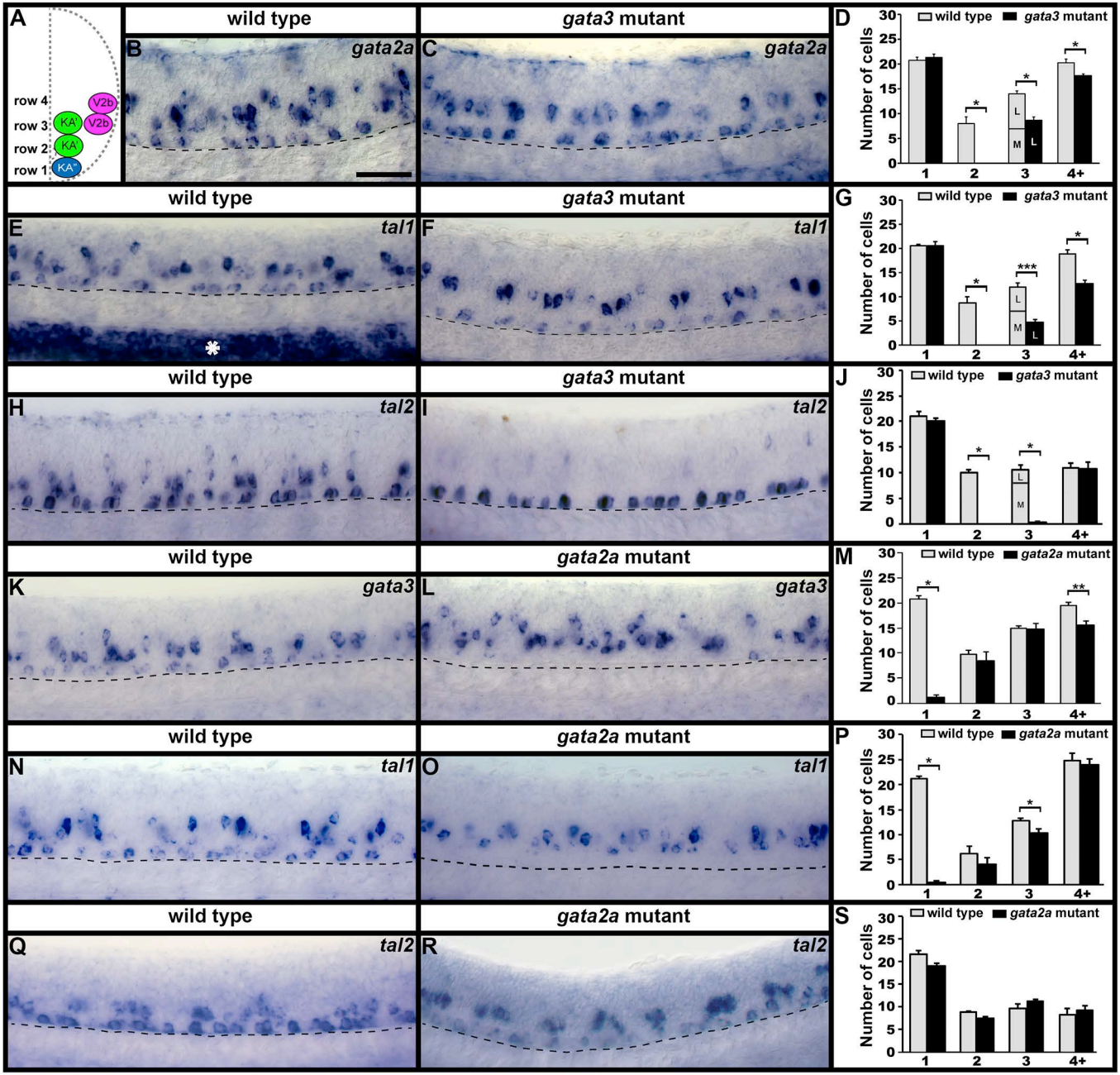
Figure 7. Neurotransmitter and neuropeptide phenotypes in *tal1*, *gata3* and *gata2a* mutants.

Lateral views of *gad*, *sst1.1* and *urp1* expression in 24h WT sibling (A, D, G, J, L, N) and *tal1* (B, K), *gata3* (E, M) and *gata2a* (H, O) mutants. Dorsal, top; anterior, left. Cross sectional schematic indicating positions of KA", KA' and V2b neurons (P). Scale bars (A) = 50 microns (A-H); (J) = 50 microns (J-O). Dashed lines indicate ventral limit of spinal cord. Mean number of cells expressing *gad* in each D/V row of spinal cord region adjacent to somites 6-10 in WT and mutant embryos (C, F, I). For *tal1* and *gata3* mutant results (C & F) the approximate proportions of medial and lateral row 3 cells are indicated by horizontal lines separating the number of medially-located cells (bottom and indicated with an "M") from the number of laterally-located cells (top and indicated with a "L"). All of the remaining row 3 cells in these mutants are lateral, consistent with them being V2b cells. More dorsal (row 4 and above) cells expressing *gad* in *tal1* mutants (B) are only weakly stained and are in a different focal plane to the strongly stained KA'' cells, so they are harder to see. All counts are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. *** indicates P<0.001; ** indicates P<0.01;* indicates P<0.05. For P values see Supp. Table 3.

Figure 8. Expression of *pkd2l1* in *tal1*, *gata3* and *gata2a* mutants.

Lateral views of *pkd2l1* expression in 24h WT sibling (A) and *tal1* (B), *gata3* (C) and *gata2a* (D) mutants. Dorsal, top; anterior, left. Scale bar = 50 microns. Dashed lines indicate ventral limit of spinal cord. The approximate proportions of medial and lateral row 3 cells are indicated by horizontal lines separating the number of medially-located cells (bottom and indicated with an "M") from the number of laterally-located cells (top and indicated with a "L"). Mean number of cells expressing *pkd2l1* in each D/V row of spinal cord region adjacent to somites 6-10 in WT and mutant embryos (E-G). All counts are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. *** indicates P<0.001; ** indicates P<0.01. For P values see Supp. Table 3. Cross sectional schematic indicating positions of KA", KA' and V2b neurons (H).

Figure 3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row 4
row 3
row 2
row 1
V2b
KA'
V2b
KA'
KA''
wild type
gata3 mutant
B
gata2a
C
gata2a
D
wild type
gata3 mutant
Number of cells
L
M
L
wild type
gata3 mutant
E
tal1
F
tal1
G
wild type
gata3 mutant
Number of cells
wild type
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wild type
gata2a mutant
Q
tal2
R
tal2
S
wild type
gata2a mutant
Number of cells
1
2
3
4+

Figure 4

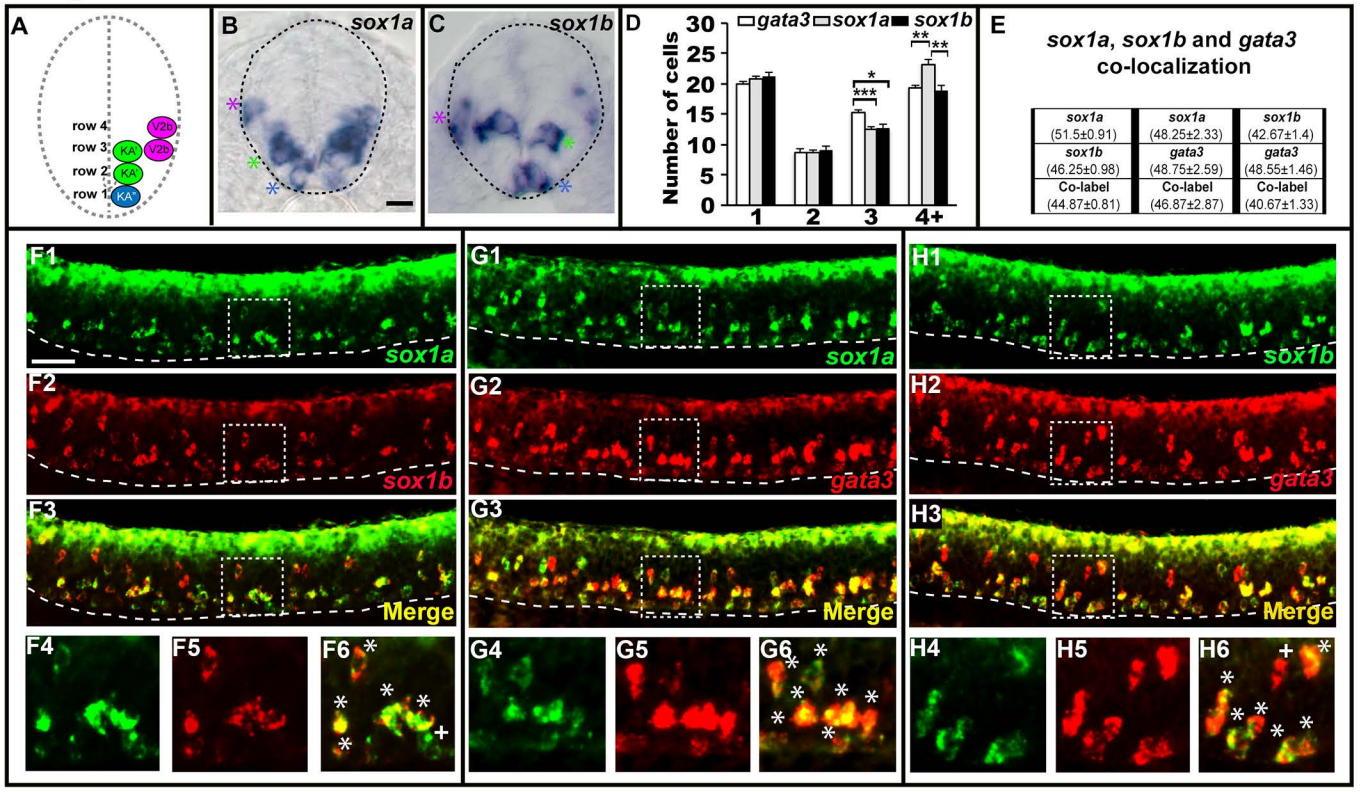

A
row 4
row 3
row 2
row 1
V2b
KA'
V2b
KA
KA"
B
sox1a
C
sox1b
D
gata3
sox1a
sox1b
Number of cells
30
25
20
15
10
5
0
1
2
3
4+
*
***
**
**
E
sox1a, sox1b and gata3
co-localization
sox1a (51.5±0.91)
sox1a (48.25±2.33)
sox1b (42.67±1.4)
sox1b (46.25±0.98)
gata3 (48.75±2.59)
gata3 (48.55±1.46)
Co-label (44.87±0.81)
Co-label (46.87±2.87)
Co-label (40.67±1.33)
F1
sox1a
F2
sox1b
F3
Merge
F4
F5
F6
G1
sox1a
G2
gata3
G3
Merge
G4
G5
G6
H1
sox1b
H2
gata3
H3
Merge
H4
H5
H6

Figure 5

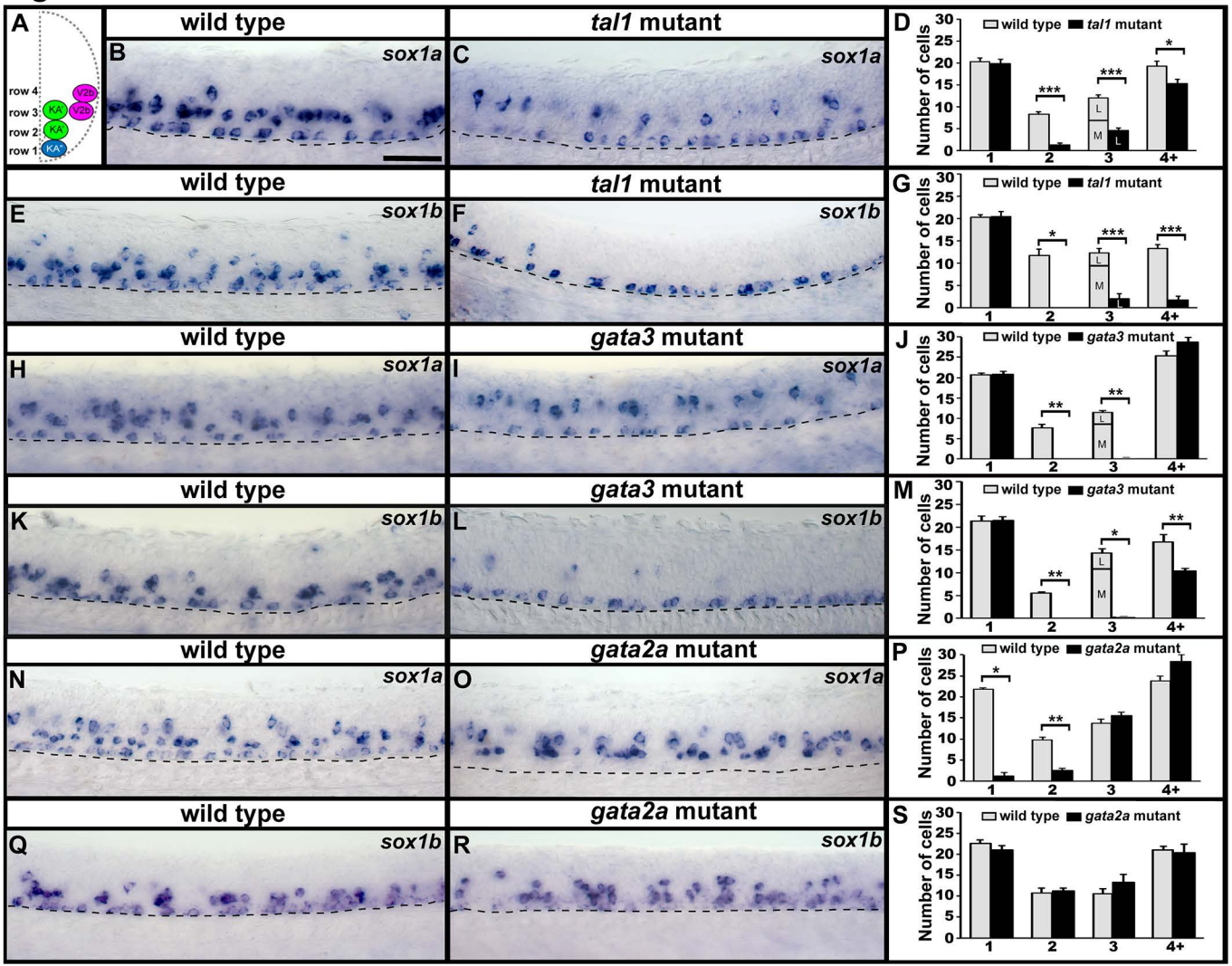

A
row 4
row 3
row 2
row 1
V2b
KA'
V2b
KA
KA'
wild type
sox1a
B
tal1 mutant
sox1a
C
D
wild type
tal1 mutant
Number of cells
L
M
L
1
2
3
4+
wild type
sox1b
E
tal1 mutant
sox1b
F
G
wild type
tal1 mutant
wild type
sox1a
H
gata3 mutant
sox1a
I
J
wild type
gata3 mutant
wild type
sox1b
K
gata3 mutant
sox1b
L
M
wild type
gata2a mutant
wild type
sox1a
N
gata2a mutant
sox1a
O
P
wild type
gata2a mutant
wild type
sox1b
Q
gata2a mutant
sox1b
R
S
wild type
gata2a mutant

Figure 6

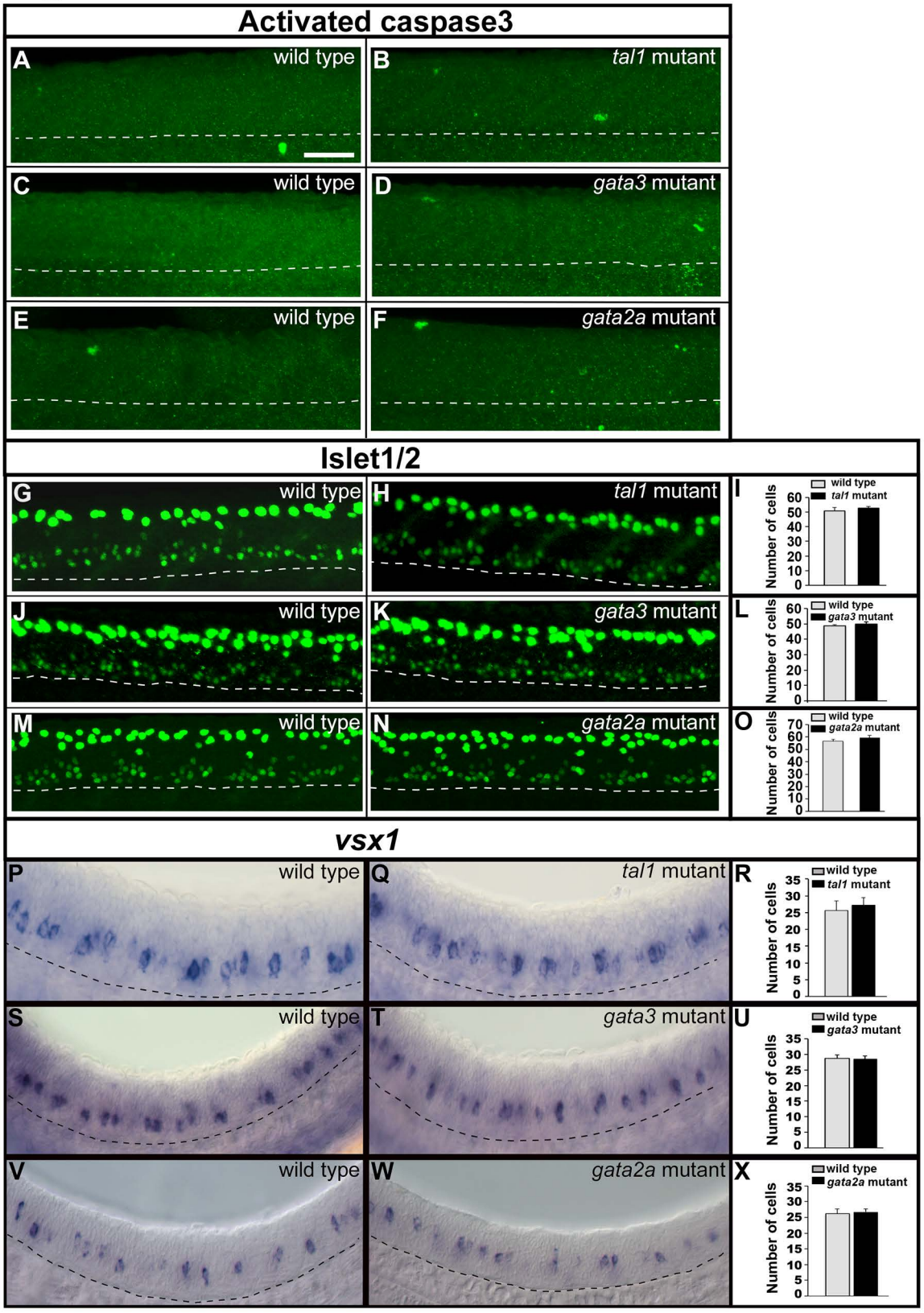

Activated caspase3
A
wild type
B
tal1 mutant
C
wild type
D
gata3 mutant
E
wild type
F
gata2a mutant
Islet1/2
G
wild type
H
tal1 mutant
I
wild type
tal1 mutant
Number of cells
J
wild type
K
gata3 mutant
L
wild type
gata3 mutant
Number of cells
M
wild type
N
gata2a mutant
O
wild type
gata2a mutant
Number of cells
vsx1
P
wild type
Q
tal1 mutant
R
wild type
tal1 mutant
Number of cells
S
wild type
T
gata3 mutant
U
wild type
gata3 mutant
Number of cells
V
wild type
W
gata2a mutant
X
wild type
gata2a mutant
Number of cells

Figure 7

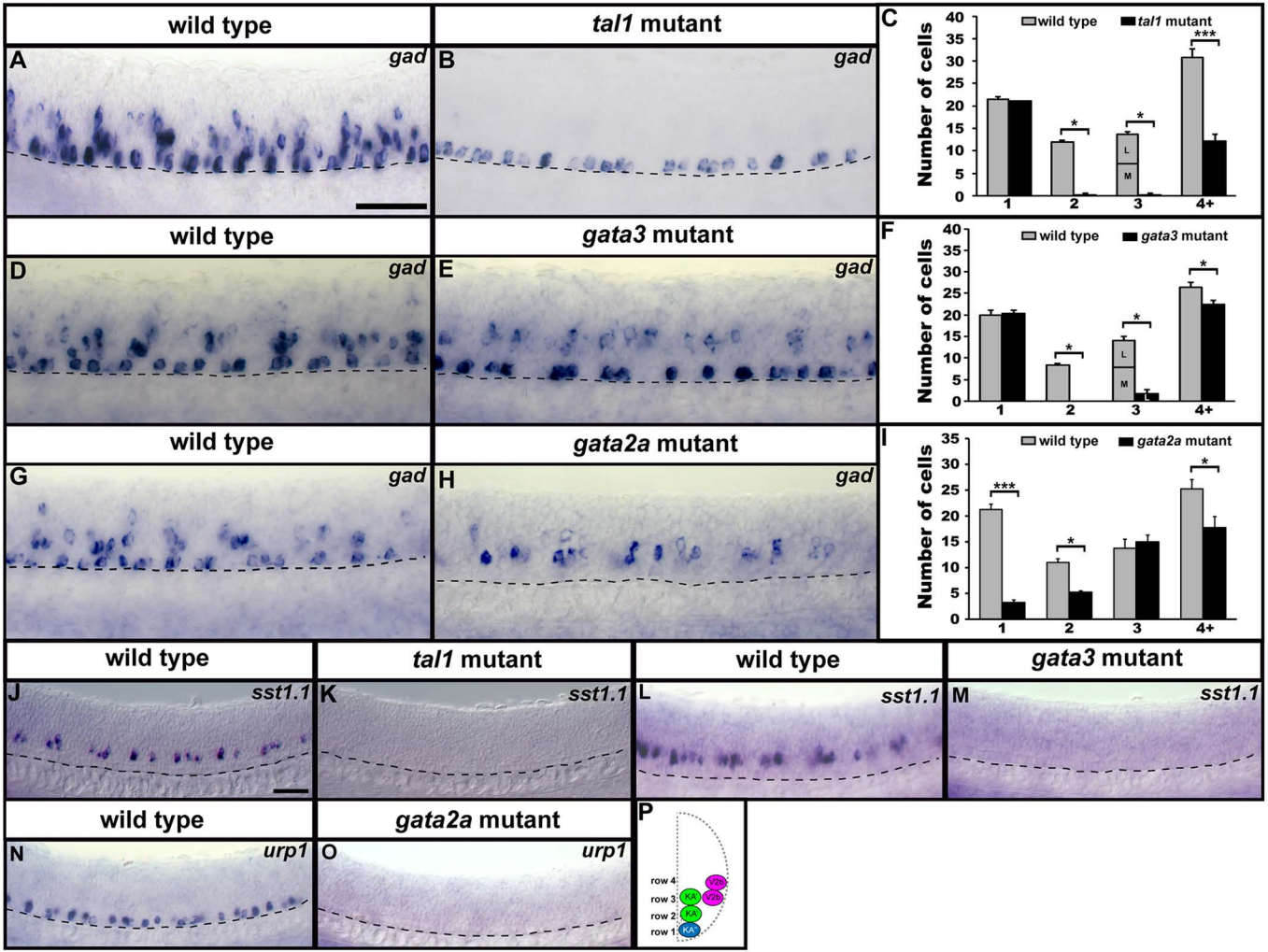

wild type
tal1 mutant
A
gad
B
gad
C
wild type
tal1 mutant
Number of cells
40
35
30
25
20
15
10
5
0
*
*
***
L
M
1
2
3
4+
wild type
gata3 mutant
D
gad
E
gad
F
wild type
gata3 mutant
Number of cells
*
*
*
L
M
1
2
3
4+
wild type
gata2a mutant
G
gad
H
gad
I
wild type
gata2a mutant
Number of cells
35
30
25
20
15
10
5
0
***
*
*
1
2
3
4+
wild type
tal1 mutant
wild type
gata3 mutant
J
sst1.1
K
sst1.1
L
sst1.1
M
sst1.1
wild type
gata2a mutant
N
urp1
O
urp1
P
row 4
row 3
row 2
row 1
KA
V2a
KA
V2a
KA
KA'

Figure 8

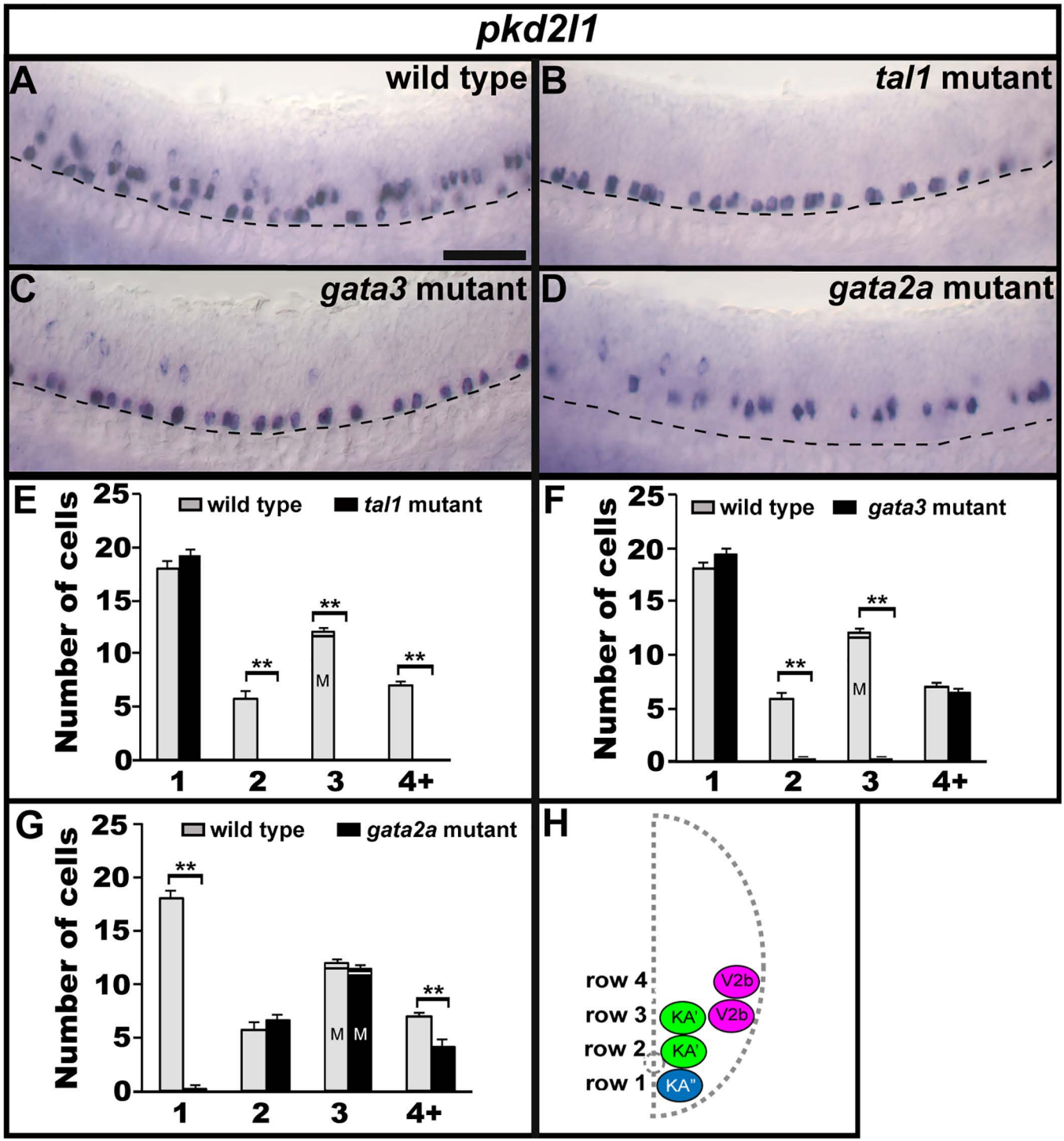

pkd2l1
A
wild type
B
tal1 mutant
C
gata3 mutant
D
gata2a mutant
E
wild type
tal1 mutant
Number of cells
25
20
15
10
5
0
**
**
**
M
1
2
3
4+
F
wild type
gata3 mutant
Number of cells
**
**
M
1
2
3
4+
G
wild type
gata2a mutant
Number of cells
**
**
M
M
1
2
3
4+
H
row 4
row 3
row 2
row 1
V2b
KA'
V2b
KA'
KA"

Figure 9. Expression of *Tg(-8.1gata1:gata1-EGFP)* in single and double mutants.

Lateral views of 30h *Tg(-8.1gata1:gata1-EGFP)* WT sibling (A) and single and double mutants as indicated (B-F). Dorsal, top; anterior, left. Dashed rectangle regions in main panels (A-F) are shown in magnified view in adjacent (A'-F') panels. Dashed lines indicate ventral limit of spinal cord. White arrows indicate KA" axons, magenta arrows indicate KA' axons and white arrowheads mark V2b axons. KA'' and KA' axons are ipsilateral and ascending whereas V2b axons are ipsilateral descending. KA'' neurons appear to form normally and extend WT-like axonal projections in *tal1;gata3* double mutants (E, E'). In contrast, even though GFP-expressing V2b cells are present in both *gata3* (C, C') and *gata2a* (D, D') single mutants there are no GFP-expressing V2b cells in *gata2a;gata3* double mutants (F, F'). Scale bar= 50 microns.

Figure 10. *gad* and *pkd2l1* expression in double mutant embryos.

Lateral views of *gad* (A, B, C) or *pkd2l1* (F, G) expression in 24h WT sibling and double mutant embryos as indicated. Dorsal, top; anterior, left. Scale bar = 50 microns. Dashed lines indicate ventral limit of spinal cord. The approximate proportions of medial and lateral row 3 cells are indicated by horizontal lines separating the number of medially-located cells (bottom and indicated where there is space with an "M") from the number of laterally-located cells (top and indicated where there is space with a "L"). Mean number of cells expressing *gad* or *pkd2l1* in each D/V row of precisely-defined spinal cord region adjacent to somites 6-10 in WT and single and double mutant embryos (D, E, H). All counts are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. *** indicates $P<0.001$; ** indicates $P<0.01$. In (E) there is no statistically significant difference between any row 1 values or between *tal1* single mutants and *tal1;gata3* double mutants in row 4+. In (H) there is no statistically significant difference between any row 1 values. For P values see Supp. Table 3. Cross sectional schematic indicating positions of KA", KA' and V2b neurons (I).

Figure 11. Expression of *slc17a6* and *sim1* in mutant embryos.

Lateral views of *slc17a6a/b (vglut)* (A, B, D, E, G, H, J, K), *slc17a6a/b* (green) and *sox1b* (red) (M) and *sim1a* (N, O) expression in WT sibling and mutant embryos as indicated. Dorsal, top; anterior, left. All embryos are 24h except the WT embryo in M which is 32h. Arrowheads indicate glutamatergic cells (H, J and K) or *sim1a*-expressing cells (N, O) in the KA'' domain. Scale bar= 50 microns. Dashed lines indicate ventral limit of spinal cord. White boxes in (M) are single confocal magnified views of dotted white box. White stars indicate double-labeled cells. Mean number of cells expressing these

genes in spinal cord region adjacent to somites 6-10 in WT and mutant embryos (C, F, I, L, P). C, F and I show counts for all dorsal-ventral rows. L and P show counts for just row 1. All counts are an average of at least 4 embryos. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. ** indicates P<0.01; * indicates P<0.05. For P values see Supp. Table 6.

Figure 12. Phospho-histone H3 (pH3) immunohistochemistry in *tal1*, *gata3* and *gata2a* mutants.
Lateral views of pH3 (red) and GFP (green) immunohistochemistry in 24h *Tg(-8.1gata1:gata1-EGFP)* WT sibling and mutant embryos as indicated. Dorsal, top; anterior, left. Scale bar = 50 microns. Dashed lines indicate ventral limit of spinal cord. Mean number of pH3-positive cells in each D/V row of spinal cord region adjacent to somites 6-10 in WT and mutant embryos (C, F, I). All counts are an average of at least 3 embryos. Cell rows were assigned based on average cell diameters. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. * indicates P<0.05. For P values see Supp. Table 6.

Figure 13. Expression of pH3 and either *olig2* or Nkx6.1 in *tal1* and *gata3* mutants.
Lateral (A, B, F, G, K, L) and cross-sectional (C, D, H, I, M, N) views of pH3 (red) and either *olig2* or Nkx6.1 (green) expression in WT embryos (A, C, F, H, K, M), *gata3* mutants (B, D, L, N) or *tal1* mutants (G, I) at 24h. Dorsal, top; in lateral views, anterior, left. White boxes in top right hand corners of lateral views are single confocal plane magnified views of the area indicated with a white dotted box. White stars indicate double-labeled cells. White dashed lines indicate the ventral limit of the spinal cord (lateral views) or the boundary of the spinal cord (cross sections). For lateral views single channel and merged images are shown. Scale bar = 50 microns. Mean number of pH3-positive; *olig2*-negative cells (E1 and J1) and pH3-positive; *olig2*-positive cells (E2 and J2) adjacent to somites 6-10 in WT and mutant embryos. Mean number of pH3-positive; Nkx6.1-negative cells (O1) and pH3-positive; Nkx6.1-positive cells (O2) adjacent to somites 6-10 in WT and mutant embryos. All counts are an average of at least 3 embryos. Cell rows were assigned based on average cell diameters. Error bars indicate SEM. Statistically significant comparisons are indicated with brackets and stars. * indicates P<0.05. For P values see Supp. Table 6.

Supplementary Figures and Tables

Legends for supplementary Figures 1 and 2 and all tables are provided in the separate supplementary data file.

Figure 9

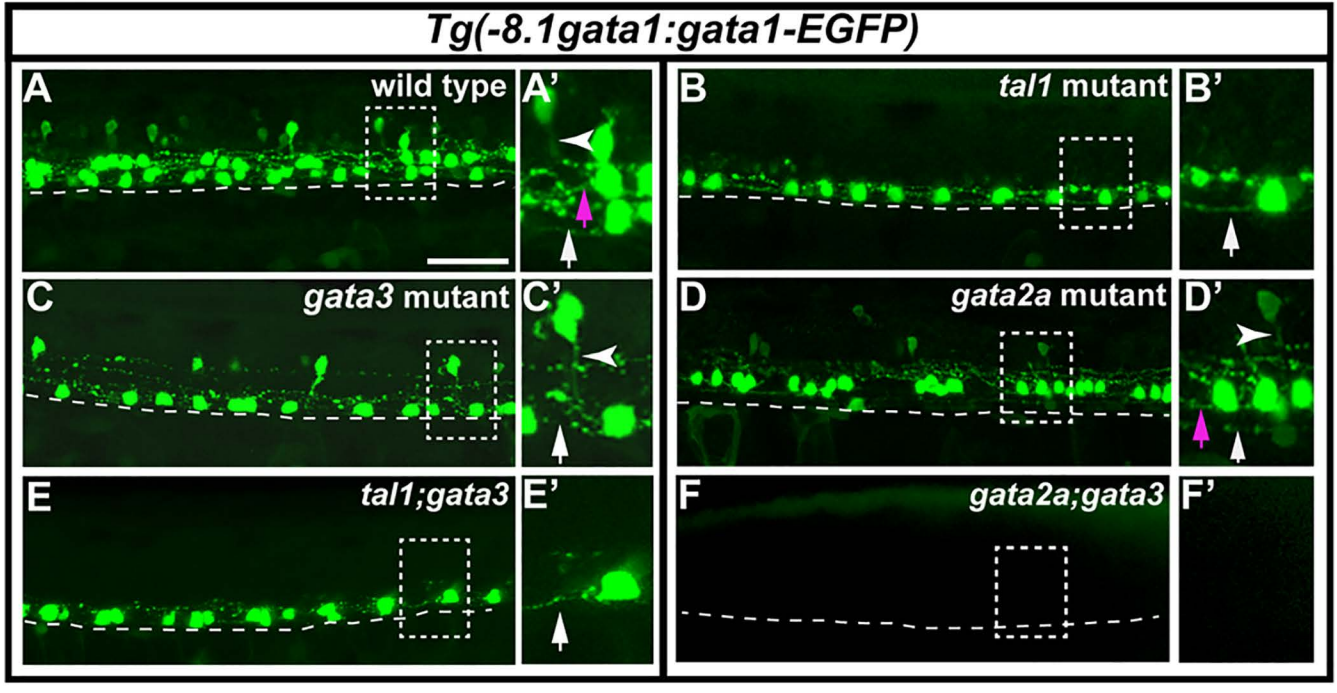

Tg(-8.1gata1:gata1-EGFP)
A
wild type
A'
B
tal1 mutant
B'
C
gata3 mutant
C'
D
gata2a mutant
D'
E
tal1;gata3
E'
F
gata2a;gata3
F'

Figure 10

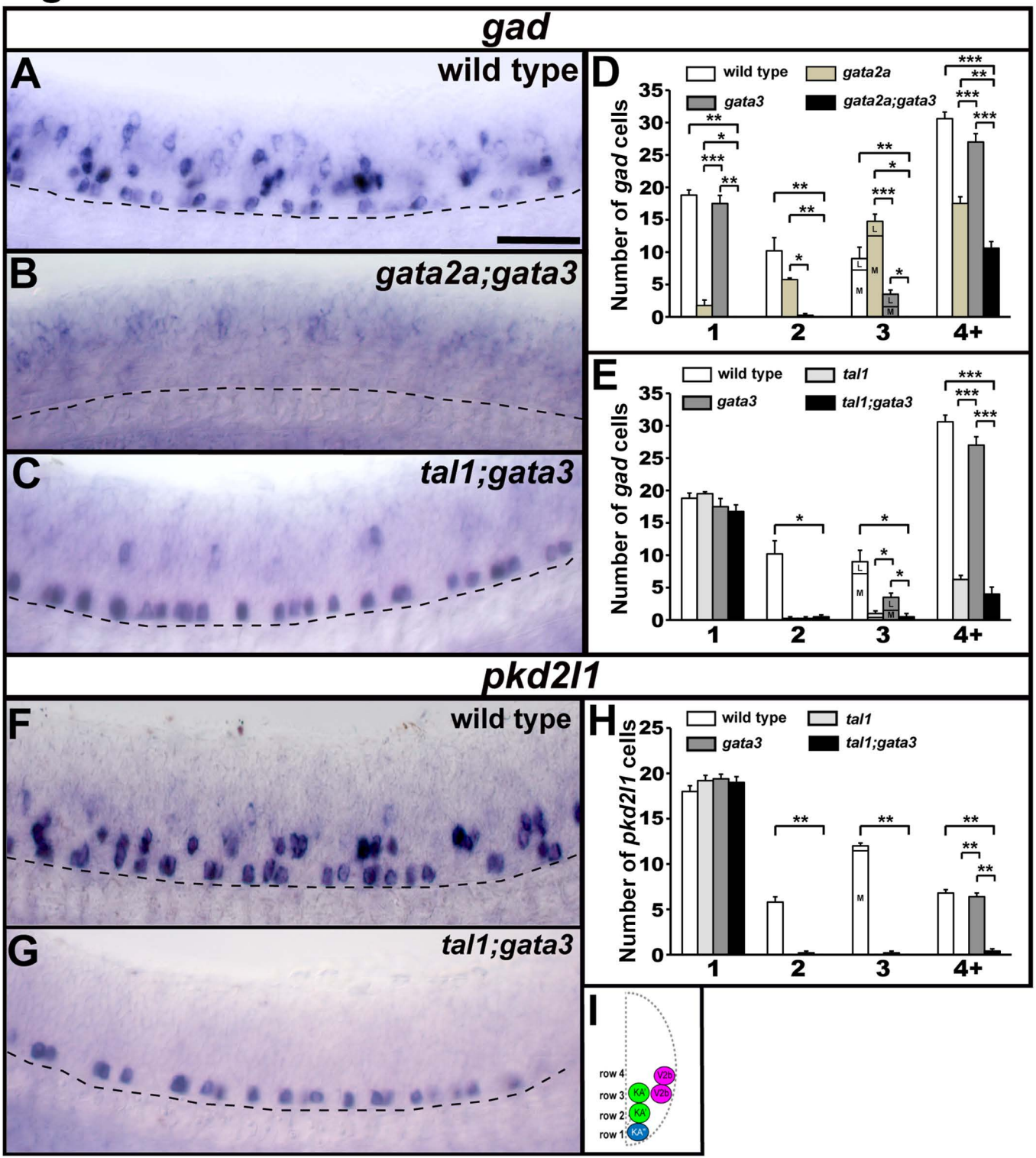

gad
A
wild type
B
gata2a;gata3
C
tal1;gata3
D
wild type
gata2a
gata3
gata2a;gata3
Number of gad cells
1
2
3
4+
E
wild type
tal1
gata3
tal1;gata3
Number of gad cells
pkd2l1
F
wild type
G
tal1;gata3
H
wild type
tal1
gata3
tal1;gata3
Number of pkd2l1 cells
I
row 4
row 3
row 2
row 1
V2b
KA
KA'

Figure 11

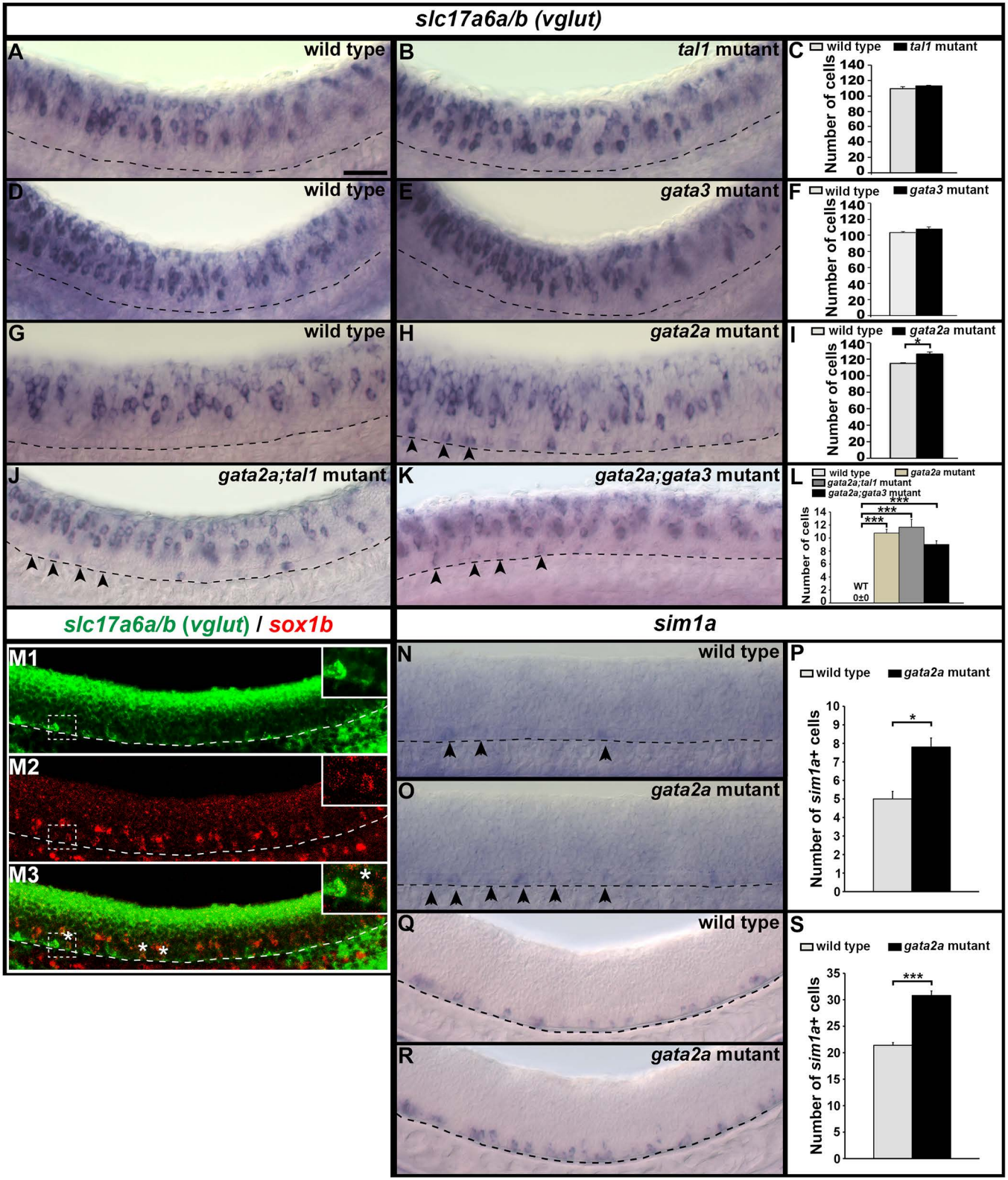

slc17a6a/b (vglut)
A
wild type
B
tal1 mutant
C
wild type
tal1 mutant
Number of cells
140
120
100
80
60
40
20
0
D
wild type
E
gata3 mutant
F
wild type
gata3 mutant
Number of cells
G
wild type
H
gata2a mutant
I
wild type
gata2a mutant
*
Number of cells
J
gata2a;tal1 mutant
K
gata2a;gata3 mutant
L
wild type
gata2a mutant
gata2a;tal1 mutant
gata2a;gata3 mutant
***
***
***
***
Number of cells
14
12
10
8
6
4
2
0
WT
0±0
slc17a6a/b (vglut) / sox1b
M1
M2
M3
*
*
*
sim1a
N
wild type
O
gata2a mutant
P
wild type
gata2a mutant
*
Number of sim1a+ cells
10
9
8
7
6
5
4
3
2
1
0
Q
wild type
R
gata2a mutant
S
wild type
gata2a mutant
***
Number of sim1a+ cells
35
30
25
20
15
10
5
0

Figure 12

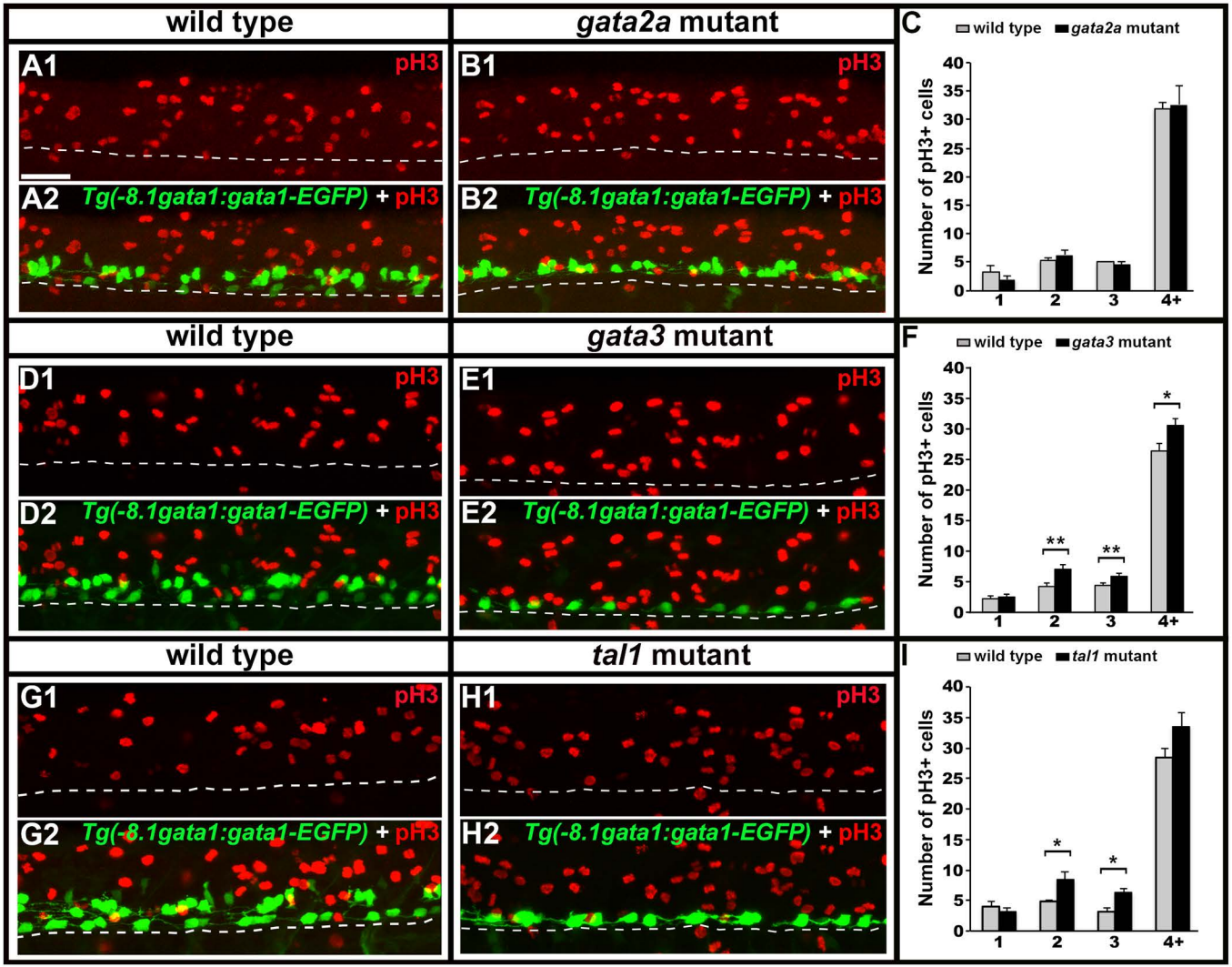
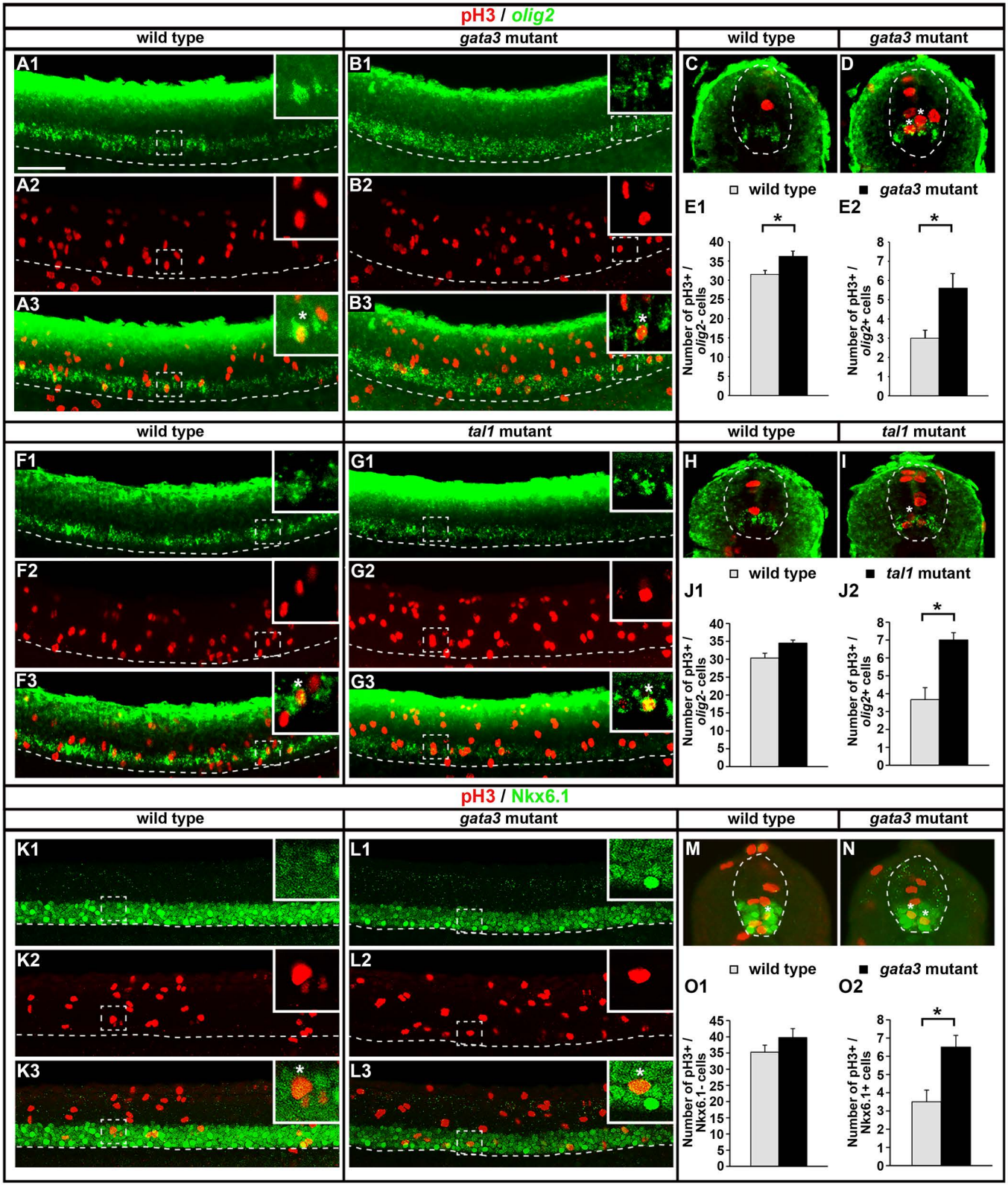

wild type
gata2a mutant
A1
pH3
B1
pH3
A2
Tg(-8.1gata1:gata1-EGFP) + pH3
B2
Tg(-8.1gata1:gata1-EGFP) + pH3
C
wild type
gata2a mutant
Number of pH3+ cells
40
35
30
25
20
15
10
5
0
1
2
3
4+
wild type
gata3 mutant
D1
pH3
E1
pH3
D2
Tg(-8.1gata1:gata1-EGFP) + pH3
E2
Tg(-8.1gata1:gata1-EGFP) + pH3
F
wild type
gata3 mutant
Number of pH3+ cells
**
**
*
1
2
3
4+
wild type
tal1 mutant
G1
pH3
H1
pH3
G2
Tg(-8.1gata1:gata1-EGFP) + pH3
H2
Tg(-8.1gata1:gata1-EGFP) + pH3
I
wild type
tal1 mutant
Number of pH3+ cells
*
*
1
2
3
4+

Figure 13


pH3 / olig2
wild type
gata3 mutant
wild type
gata3 mutant
A1
A2
A3
B1
B2
B3
C
D
wild type
gata3 mutant
E1
E2
Number of pH3+ / olig2- cells
Number of pH3+ / olig2+ cells
wild type
tal1 mutant
wild type
tal1 mutant
F1
F2
F3
G1
G2
G3
H
I
wild type
tal1 mutant
J1
J2
Number of pH3+ / olig2- cells
Number of pH3+ / olig2+ cells
pH3 / Nkx6.1
wild type
gata3 mutant
wild type
gata3 mutant
K1
K2
K3
L1
L2
L3
M
N
wild type
gata3 mutant
O1
O2
Number of pH3+ / Nkx6.1- cells
Number of pH3+ / Nkx6.1+ cells

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1246

Supplementary Material

Tal1, Gata2a and Gata3 have distinct functions in the development of V2b and cerebrospinal fluid-contacting KA spinal neurons

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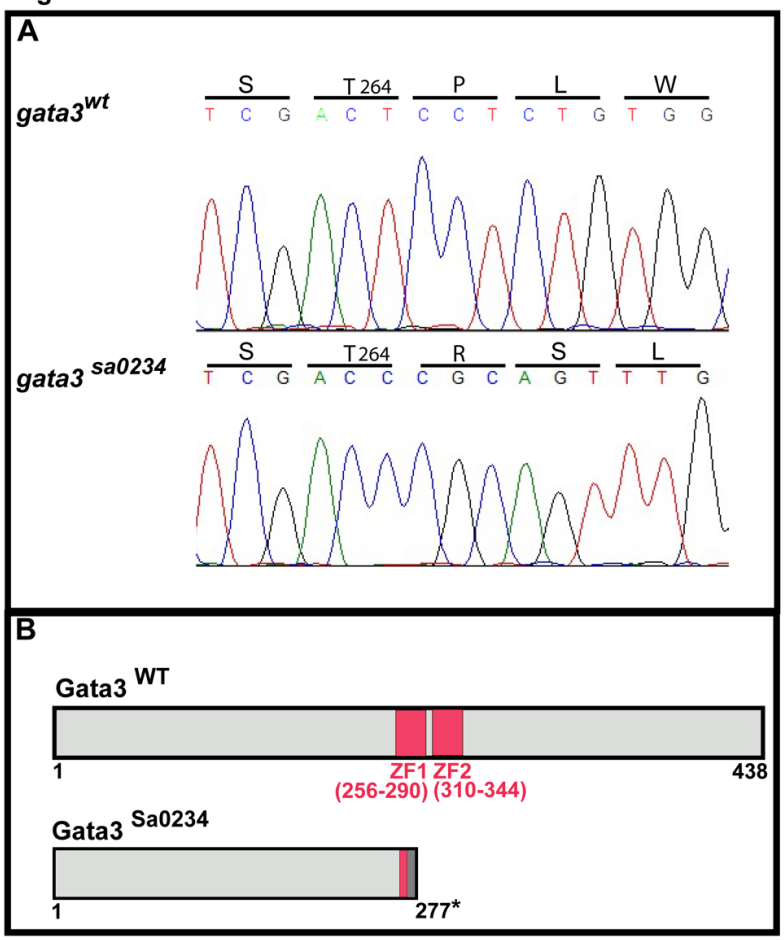
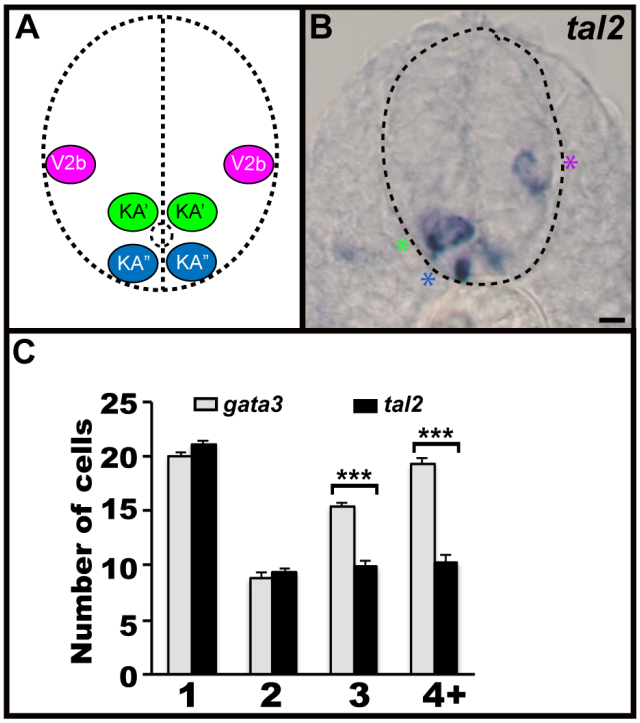
Figure S1



Supplementary Figure 1. *gata3*^{sa0234} mutant allele encodes a truncated protein that lacks both zinc finger domains.

(A) Electropherogram of *gata3* WT and *sa0234* mutant alleles with nucleotides, and single letter codes for corresponding amino acids, indicated above each trace. (B) WT Gata3 contains 438 amino acids and two zinc fingers (ZF1 and ZF2) indicated in red. Gata3^{sa0234} protein is truncated after 277 amino acids and has 13 aberrant amino acids (shaded in dark grey) following Threonine 264 (shown in A). Although Gata3^{sa0234} contains the first 8 amino acids of ZF1, EMBL-pfam (pfam.xfam.org/) failed to identify any zinc finger domains in this protein.

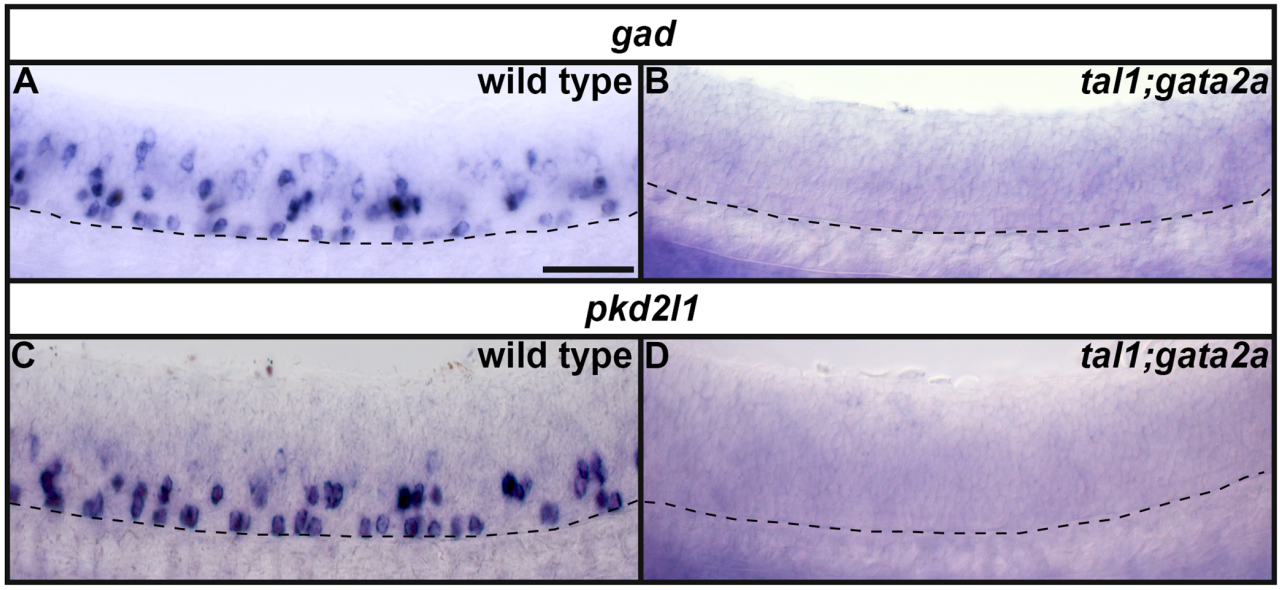
Figure S2



Supplementary Figure 2. *tal2* is expressed in all KA neurons and a few V2b neurons.

Cross-sectional (A-B) views of 24h WT zebrafish embryos; dorsal, top. (A) Schematic indicating positions of KA", KA' and V2b neurons. (B) *tal2* expression in KA" (blue asterisk), KA' (green asterisk) and V2b (magenta asterisk) cells. Dotted line (B) shows spinal cord boundary. The spacing / rostral-caudal position of KA'', KA' and V2b cells is not identical on the two sides of the spinal cord. In this particular cross section there is a V2b cell only on the right hand side and there are more KA neurons on the left hand side than on the right hand side. Scale bar = 10 microns. (C) Mean number of cells expressing *gata3* or *tal2* in each D/V row of spinal cord region adjacent to somites 6-10. All counts are an average of at least 5 embryos. Error bars indicate SEM. Statistically significant ($P<0.001$) comparisons are indicated with square brackets and three stars (***). Compared to *gata3* expression, there are statistically significantly fewer cells expressing *tal2* in row 3 and above, suggesting that *tal2* is only expressed in a subset of V2b cells. For P values see Table 3.

Figure S3



Supplementary Figure 3. *gad* and *pkd2l1* expression in *tal1;gata2a* double mutants.

Lateral views of *gad* (A, B) or *pkd2l1* (C, D) expression in 24h WT sibling (A, C) and *tal1;gata2a* double mutant embryos (B, D) as indicated. Dorsal, top; anterior, left. Scale bar = 50 microns. Dashed lines indicate ventral limit of spinal cord.

Supplementary Table 1: Primer sequences for PCR *in situ* probe generation.

Gene	PCR primers
gata2a	Forward- GTGAGGGTTTCGAGGAGCTC Reverse- **AATTAACCCTCACTAAAGGGA**AGCAACATCGCCTTGGCTAG
gata3	Forward– CTGCTACCTCCAATCTCCCAC Reverse-***AATTAACCCTCACTAAAGGGA***CAACCATTCAGTCTGCATTACATAAAG
sst1.1	Forward- AAGATGCTCTCCACGCGTAT Reverse- ***AATTAACCCTCACTAAAGGGA***AGCAGATTTGCACACATTCG
urp1	Forward- AAGTCAGCCACGATCCTCTCAAC Reverse- ***AATTAACCCTCACTAAAGGGA***AATGAGGGTGTGTTAGGCTGGTC

PCR primers used to amplify *in situ* hybridization probe templates for genes indicated in the left hand column. In all cases, reverse primers contained the T3 RNA polymerase promoter-binding site (bold italics), used to synthesize antisense RNA probe. Probes were PCR amplified from 27h WT zebrafish cDNA (see materials and methods).

Supplementary Table 2: Gene names, previous names, ZFIN identifiers and references for *in situ* hybridization probes used**.**

Gene name	ZFIN ID	Reference for probe
gata2a (previously called *gata2*)	ZDB-GENE-980526-260	This paper (see Supp. Table 1)
gata3	ZDB-GENE-990415-82	This paper (see Supp. Table 1)
tal1 (previously called *scl*)	ZDB-GENE-980526-501	(Peng *et al*., 2007)
tal2	ZDB-GENE-040115-1	(Pinheiro *et al*., 2004)
sox1a	ZDB-GENE-040718-186	(A gift from Strähle lab from library described in Armant *et al.*, 2013)
sox1b	ZDB-GENE-060322-5	(A gift from Strähle lab from library described in Armant *et al.*, 2013)
gad2 (previously called *gad65*)	ZDB-GENE-030909-9	(Higashijima *et al*., 2004b & c)
gad1b (previously called *gad67*)	ZDB-GENE-030909-3	(Higashijima *et al*., 2004b & c)
slc17a6a (previously called *vglut 2.2*)	ZDB-GENE-050105-4	(Higashijima *et al*., 2004b & c)
slc17a6b *(*previously called *vglut2.1)*	ZDB-GENE-030616-554	(Higashijima *et al*., 2004b & c)
pkd2l1	ZDB-GENE-030616-558	(England *et al*., 2017)
sst1.1	ZDB-GENE-030131-4743	This paper (see Supp. Table 1)
urp1	ZDB-GENE-100922-138	This paper (see Supp. Table 1)
sim1a	ZDB-GENE-020829-1	(Schäfer *et al*., 2007)
olig2	ZDB-GENE-030131-4013	(Park *et al*., 2002)

Column 1 lists genes used in this study, along with previous names where appropriate. Column 2 provides the unique ZFIN identification number for each gene. Column 3 indicates the reference for the *in situ* hybridization probe used in our experiments.

Supplementary Table 3. Statistical comparisons of numbers of cells expressing particular genes.

Figure	Comparison	Gene	Row 1 cells	Row 2 cells	Row 3 cells	Row 4+ cells
1E	*gata3* vs *tal1* in WT	N/A	0.523	0.725	**<0.001**	**<0.001**
1H	WT vs *tal1* mutant	*gata3*	0.083	**<0.001**	**<0.001**	0.187
1K	WT vs *tal1* mutant	*gata2a*	0.098	**0.003**	**0.005**	0.690
1N	WT vs *tal1* mutant	*tal2*	0.072	**<0.001**	**0.001**	**0.001**
3D	WT vs *gata3* mutant	*gata2a*	0.349	**<0.001**	**0.004**	**0.033**
3G	WT vs *gata3* mutant	*tal1*	1.000	**<0.001**	**<0.001**	**0.006**
3J	WT vs *gata3* mutant	*tal2*	0.422	**<0.001**	**0.001**	0.877
3M	WT vs *gata2a* mutant	*gata3*	**<0.001**	0.521	0.868	**0.007**
3P	WT vs *gata2a* mutant	*tal1*	**<0.001**	0.298	0.539	0.706
3S	WT vs *gata2a* mutant	*tal2*	0.059	0.061	0.2446	0.613
4D	*gata3* vs *sox1a* in WT	N/A	0.761	0.817	**<0.001**	**0.001**
4D	*gata3* vs *sox1b* in WT	N/A	0.596	0.781	**0.006**	0.751
4D	*sox1a* vs *sox1b* in WT	N/A	0.311	0.350	0.429	**0.003**
5D	WT vs *tal1* mutant	*sox1a*	0.734	**<0.001**	**<0.001**	**0.039**
5G	WT vs *tal1* mutant	*sox1b*	0.955	**0.001**	**0.001**	**<0.001**
5J	WT vs *gata3* mutant	*sox1a*	0.057	**0.001**	**<0.001**	0.085
5M	WT vs *gata3* mutant	*sox1b*	0.922	**<0.001**	**<0.001**	**0.026**
5P	WT vs *gata2a* mutant	*sox1a*	**<0.001**	**<0.001**	0.182	0.055
5S	WT vs *gata2a* mutant	*sox1b*	0.322	0.723	0.280	0.759
7C	WT vs *tal1* mutant	*gad*	0.391	**<0.001**	**<0.001**	**<0.001**
7F	WT vs *gata3* mutant	*gad*	0.874	**<0.001**	**<0.001**	0.062
7I	WT vs *gata2a* mutant	*gad*	**<0.001**	**0.002**	0.588	**0.037**
8E	WT vs *tal1* mutant	*pkd2l1*	0.200	**<0.001**	**<0.001**	**<0.001**
8F	WT vs *gata3* mutant	*pkd2l1*	0.229	**<0.001**	**<0.001**	0.330
8G	WT vs *gata2a* mutant	*pkd2l1*	**<0.001**	0.332	0.275	**0.003**
10D	WT vs *gata2a;gata3* double mutants	*gad*	**<0.001**	**0.008**	**0.007**	**<0.001**
10D	*gata2a* mutants vs *gata2a;gata3* double mutants	*gad*	0.133	**<0.001**	**<0.001**	**0.002**
10D	*gata3* mutants vs *gata2a;gata3* double mutants	*gad*	**<0.001**	0.391	**<0.001**	**<0.001**
10D	*gata2a* mutants vs *gata3* mutants	*gad*	**<0.001**	**<0.001**	**<0.001**	**0.002**
10E	WT vs *tal1;gata3* double mutants	*gad*	0.167	**0.008**	**0.007**	**<0.001**
10E	*tal1* mutants vs *tal1;gata3* double mutants	*gad*	0.071	0.537	0.469	0.134
10E	*gata3* mutants vs *tal1;gata3* double mutants	*gad*	0.421	0.537	**0.012**	**<0.001**
10E	*gata3* mutants vs *tal1* mutants	*gad*	0.211	1	**0.022**	**<0.001**
10H	WT vs *tal1;gata3* double mutants	*pkd2l1*	0.255	**<0.001**	**<0.001**	**<0.001**

10H	*gata3* mutants vs *tal1;gata3* double mutants	*pkd2l1*	0.636	0.374	0.374	<**0.001**
10H	*tal1* mutants vs *tal1;gata3* double mutants	*pkd2l1*	0.822	ND	ND	ND
10H	*tal1* mutants vs *gata3* mutants	*pkd2l1*	0.803	0.374	0.374	<**0.001**
12C	WT vs *gata2a* mutants	pH3	0.394	0.379	0.423	0.863
12F	WT vs *gata3* mutants	pH3	0.601	**0.002**	**0.008**	**0.013**
12I	WT vs *tal1* mutants	pH3	0.496	**0.049**	**0.019**	0.129
S2C	*gata3* vs *tal2* in WT	N/A	0.457	0.600	<**0.001**	<**0.001**

Statistical comparisons between the number of cells expressing particular genes using Student's t-test. First column indicates the figure that shows the relevant bar chart for the comparison (S = Supplementary Figure). Second column states what the comparison is. Where embryos of different genotypes are being compared, column three states the gene that the comparison refers to. If WT embryos are being compared then N/A (not applicable) is listed in column 3. The last four columns show the P values for the comparisons for each row shown in the corresponding bar chart. Values are rounded up to three decimal places. P values less than 0.001 are listed as <0.001. Statistically significant ($P<0.05$) values are indicated in bold. For double mutant comparisons P values are only listed for comparisons with the double mutants and between the single mutants. Other comparisons can be found in earlier single mutant studies. For figure 10H, P values for rows 2, 3, and 4+ could not be determined (ND) as all cell counts were zero in both *tal1* and *tal1;gata3* double mutants).

Supplementary Table 4. Cell type specific phenotypes in *tal1* mutants.

tal1 mutant		Row #	1	2	3M	3L	4+
		Cells	KA"	KA'		V2b	
gata3	WT	Rows	21.25	7.00	7.75	7.25	18.0
		Cells	21.25	14.75		25.25	
	MUT	Rows	19.00	1.67	0.00	2.33	15.00
		Cells	19.00	1.67		17.33	
gata2a	WT	Rows	19.50	9.85	6.75	7.80	17.5
		Cells	19.50	16.6		25.3	
	MUT	Rows	20.25	0.25	0.00	7.00	17.0
		Cells	20.25	0.25		24.00	
tal2	WT	Rows	20.50	10.75	7.25	3.50	12.50
		Cells	20.50	18.00		16.00	
	MUT	Rows	18.75	0.00	0.00	0.00	0.00
		Cells	18.75	0.00		0.00	
sox1a	WT	Rows	20.25	8.25	6.75	5.25	19.25
		Cells	20.25	15.00		24.50	
	MUT	Rows	19.75	1.25	0.00	4.50	15.25
		Cells	19.75	1.25		19.75	
sox1b	WT	Rows	20.25	11.75	9.25	3.00	13.25
		Cells	20.25	21.00		16.25	
	MUT	Rows	20.33	0.00	0.00	2.00	1.67
		Cells	20.33	0.00		3.67	
gad	WT	Rows	21.50	12.00	7.00	6.75	30.75
		Cells	21.50	19.00		37.50	
	MUT	Rows	21.00	0.25	0.00	0.25	12.25
		Cells	21.00	0.25		12.50	
pkd2l1	WT	Rows	18.00	5.80	11.40	0.60	7.00
		Cells	18.00	17.20		7.60	
	MUT	Rows	19.20	0.00	0.00	0.00	0.00
		Cells	19.20	0.00		0.00	

Gene expression phenotypes in *tal1* mutants. Every other row of the table indicates the mean number of cells expressing specific genes, indicated in the first column, in particular dorsal-ventral spinal cord rows of either WT or *tal1* mutants. Row 3 data is divided into medial (3M) and lateral (3L) cells. Cell type identities associated with cells in particular dorsal/ventral positions, is provided in table header and the number of cells falling into each of these categories is provided in every other row.

Supplementary Table 5. Cell type specific phenotypes in *gata3* mutants.

gata3 mutant		Row #	1	2	3M	3L	4+
		Cells	KA"	KA'		V2b	
gata2a	WT	Rows	20.33	9.30	7.67	6.67	20.67
		Cells	20.33	16.97		27.33	
	MUT	Rows	21.33	0.00	0.00	8.67	17.67
		Cells	21.33	0.00		26.33	
tal1	WT	Rows	20.50	8.75	6.75	5.25	18.75
		Cells	20.50	15.50		24.00	
	MUT	Rows	20.50	0.00	0.00	4.75	12.75
		Cells	20.50	0.00		17.50	
tal2	WT	Rows	21.00	10.00	6.81	3.69	11.00
		Cells	20.00	16.81		14.69	
	MUT	Rows	20.00	0.00	0.25	0.00	10.75
		Cells	20.00	0.25		10.75	
sox1a	WT	Rows	20.60	7.60	6.80	4.60	25.40
		Cells	20.60	14.40		30.00	
	MUT	Rows	20.83	0.00	0.17	0.00	28.67
		Cells	20.83	0.17		28.67	
sox1b	WT	Rows	21.25	5.50	10.77	3.48	16.75
		Cells	21.25	16.27		20.23	
	MUT	Rows	21.40	0.00	0.20	0.00	10.40
		Cells	21.40	0.20		10.40	
gad	WT	Rows	20.00	8.33	8.67	5.33	26.33
		Cells	20.00	17.00		31.67	
	MUT	Rows	20.30	0.00	0.00	1.75	22.50
		Cells	20.30	0.00		24.25	
pkd2l1	WT	Rows	18.00	5.80	11.40	0.60	7.00
		Cells	18.00	17.20		7.60	
	MUT	Rows	19.40	0.20	0.20	0.00	6.40
		Cells	19.40	0.40		6.40	

Gene expression phenotypes in *gata3* mutants. Every other row of the table indicates the mean number of cells expressing specific genes, indicated in the first column, in particular dorsal-ventral spinal cord rows of either WT or *gata3* mutants. Row 3 data is divided into medial (3M) and lateral (3L) cells. Cell type identities associated with cells in particular dorsal/ventral positions, is provided in table header and the number of cells falling into each of these categories is provided in every other row.

Supplementary Table 6. Statistical comparisons of numbers of cells expressing particular genes

Figure	Comparison	Gene/Protein	P value
6 G-H	WT (55.2 ±1) vs *tal1* mutants (53.2±1.2)	Islet1/2	0.250
6 I-J	WT (48.8±0.6) vs *gata3* mutants (50±1.5)	Islet1/2	0.217
6 K-L	WT (56.6±1.4) vs *gata2a* mutants (59.3±2)	Islet1/2	0.522
6 M-N	WT (26±0.8) vs *tal1* mutants (27.2±1.7)	*vsx1*	0.500
6 O-P	WT (28.8±0.5) vs *gata3* mutants (28.5±1)	*vsx1*	0.908
6 Q-R	WT (26±0.6) vs *gata2a* mutants (26.8±1.1)	*vsx1*	0.538
11 C	WT (109.2±2.4) vs *tal1* mutants (112.6±1)	*slc17a6a/b*	0.272
11 F	WT (103.4±1.4) vs *gata3* mutants (107.8±3)	*slc17a6a/b*	0.211
11 I	WT (115±0.5) vs *gata2a* mutants (126±2.1)	*slc17a6a/b*	**0.045**
11 L	WT (0±0) vs *gata2a* mutants (10.8±0.6)	*slc17a6a/b* row 1 only	**<0.001**
11 L	WT (0±0) vs *gata2a;tal1* double mutants (11.7±1.2)	*slc17a6a/b* row 1 only	**0.010**
11 L	WT (0±0) vs *gata2a;gata3* double mutants (9±0.6)	*slc17a6a/b* row 1 only	**0.004**
11 L	*gata2a* mutants (10.8±0.6) vs *gata2a;tal1* double mutants (11.7±1.2)	*slc17a6a/b* row 1 only	0.542
11 L	*gata2a* mutants (10.8±0.6) vs *gata2a;gata3* double mutants (9±0.6)	*slc17a6a/b* row 1 only	0.076
11 P	WT (5±0.5) vs *gata2a* mutants (9±0.5)	*sim1a*	**0.003**
13 E1	WT (30.7±0.9) vs *gata3* mutants (34.3±0.9)	pH3 +ve / *olig2* -ve	**0.042**
13 E2	WT (3±0.6) vs *gata3* mutants (6.3±0.7)	pH3 +ve / *olig2* +ve	**0.020**
13 J1	WT (30.3±1.3) vs *tal1* mutants (34.5±0.9)	pH3 +ve / *olig2* -ve	0.065
13 J2	WT (3.7±0.7) vs *tal1* mutants (7±0.4)	pH3 +ve / *olig2* +ve	**0.018**
13 O1	WT (35.3±2.2) vs *gata3* mutants (39.8±2.8)	pH3 +ve / Nkx6.1 -ve	0.252
13 O2	WT (3.5±0.7) vs *gata3* mutants (6.5±0.7)	pH3 +ve / Nkx6.1 +ve	**0.017**

Statistical comparisons between mutant and WT embryos using Student's t-test. First column indicates figure panel(s) that show either the relevant bar chart for the comparison (Figs 11 and 13) or representative images of WT and mutant embryos (Fig. 6). [As there were no statistically significant differences for any of the comparisons in Figure 6, we did not include bar charts in that figure]. Second column states which genotypes are being compared. Numbers within parentheses indicate mean numbers of cells ± S.E.M. For all cases except 11 L and 6 G-L, cells were counted in all dorsal-ventral spinal cord rows. For 11 L, cells were only counted in row 1. For 6 G-L Islet1/2 positive cells in the two most dorsal rows, which correspond to Rohon-Beard neurons, were not counted. Only ventral cells that correspond to motoneurons were counted. These also have smaller nuclei than Rohon Beard cells. Column three states the gene, protein or double labeling result that the cell counts and statistical comparison refers to. The last column shows the P value for the comparison. P values are rounded up to three decimal places. Statistically significant ($P<0.05$) values are indicated in bold.

Supplementary Table 7. Cell type specific phenotypes in double mutants

Double mutants		Row #	1	2	3M	3L	4+
		Cells	KA"	KA'		V2b	
gad	WT	Rows	18.80	10.20	7.20	1.80	30.60
		Cells	18.80	17.40		32.40	
	gata2a MUT	Rows	1.75	5.75	12.50	2.25	17.50
		Cells	1.75	18.25		19.75	
	gata3 MUT	Rows	17.50	0.25	1.50	2.00	27.00
		Cells	17.50	1.75		29.00	
	gata2a;gata3 MUT	Rows	0.00	0.00	0.00	0.00	10.60
		Cells	0.00	0.00		10.60	
gad	WT	Rows	18.80	10.20	7.20	1.80	30.60
		Cells	18.80	17.40		32.40	
	tal1 MUT	Rows	19.50	0.25	0.25	0.75	6.25
		Cells	19.50	0.50		7.00	
	gata3 MUT	Rows	17.50	0.25	1.50	2	27.0
		Cells	17.50	1.75		29.00	
	tal1;gata3 MUT	Rows	17.33	0.33	0.25	0.25	4.00
		Cells	17.33	0.58		4.25	
pkd2l1	WT	Rows	18.00	5.80	11.40	0.60	7.00
		Cells	18.00	17.20		7.60	
	tal1 MUT	Rows	19.20	0.00	0.00	0.00	0.00
		Cells	19.20	0.00		0.00	
	gata3 MUT	Rows	19.40	0.20	0.20	0.00	6.40
		Cells	19.40	0.40		6.40	
	tal1;gata3 MUT	Rows	19.00	0.00	0.00	0.00	0.00
		Cells	19.00	0.00		0.00	

Gene expression phenotypes in double mutants. Every other row of the table indicates the mean number of cells expressing specific genes, indicated in the first column, in particular dorsal-ventral spinal cord rows of either WTs or mutants. Row 3 data is divided into medial (3M) and lateral (3L) cells. Cell type identities associated with cells in particular dorsal/ventral positions, is provided in table header and the number of cells falling into each of these categories is provided in every other row.