Hmx3a Has Essential Functions in Zebrafish Spinal Cord, Ear and Lateral Line Development

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ABSTRACT Transcription factors that contain a homeodomain DNA-binding domain have crucial functions in most aspects of cellular function and embryonic development in both animals and plants. Hmx proteins are a subfamily of NK homeodomain-containing proteins that have fundamental roles in development of sensory structures such as the eye and the ear. However, Hmx functions in spinal cord development have not been analyzed. Here, we show that zebrafish (Danio rerio) hmx2 and hmx3a are coexpressed in spinal dI2 and V1 interneurons, whereas hmx3b, hmx1, and hmx4 are not expressed in spinal cord. Using mutational analyses, we demonstrate that, in addition to its previously reported role in ear development, hmx3a is required for correct specification of a subset of spinal interneuron neurotransmitter phenotypes, as well as correct lateral line progression and survival to adulthood. Surprisingly, despite similar expression patterns of hmx2 and hmx3a during embryonic development, zebrafish hmx2 mutants are viable and have no obviously abnormal phenotypes in sensory structures or neurons that require hmx3a. In addition, embryos homozygous for deleions of both hmx2 and hmx3a have identical phenotypes to severe hmx3a single mutants. However, mutating hmx2 in hypomorphic hmx3a mutants that usually develop normally, results in abnormal ear and lateral line phenotypes. This suggests that while hmx2 cannot compensate for loss of hmx3a, it does function in these developmental processes, although to a much lesser extent than hmx3a. More surprisingly, our mutational analyses suggest that Hmx3a may not require its homeodomain DNA-binding domain for its roles in viability or embryonic development.

KEYWORDS Hmx2; morpholino; dI2; interneuron; genetic compensation

HOMEOBOX-containing genes, and the homeodomain-containing transcription factors that they encode, have crucial functions in most aspects of cellular function and embryonic development in both animals and plants (Bürglin and Affolter 2016). They were also some of the first examples discovered of invertebrate developmental genes that are highly conserved in vertebrates (Carrasco et al. 1984; Gehring 1985). One important subclass of homeodomain proteins are NK proteins. NK genes are evolutionarily ancient and are part of the ANTP megacluster, which also includes Hox and Parahox genes. NK proteins have fundamental roles in the development of mesoderm, endoderm, the nervous system, and the heart in all bilaterian animals examined so far (Wotton et al. 2010; Holland 2013; Treffkorn et al. 2018), and they are also found in sponges, one of the most basal animals still alive, and potentially the sister group to all other animals (Larroux et al. 2007; Fortunato et al. 2014; Pisani et al. 2015; Simion et al. 2017).

Hmx proteins (H6 family Homeodomain proteins, previously called Nk5 or Nk5x proteins; see Supplemental Material, Table S1) are a key subfamily of NK proteins. In vertebrates there are usually three or four different Hmx genes, as Hmx4 is only found in some species (Wotton et al. 2010). Interestingly, Hmx2 and Hmx3 are usually located adjacent to each other on the same chromosome, and this is also the case for Hmx1 and Hmx4, suggesting that both pairs of genes arose from tandem duplication events rather than the two rounds of whole-genome duplication that occurred at the base of the vertebrates (Wotton et al. 2010). In
teleosts, there are occasionally extra duplicates of one or more of these genes as the result of the additional genome duplication in this lineage, although interestingly, the retained genes are not consistent between different teleost species (Wotton et al. 2010). In zebrafish there are two $hmx3$ genes, $hmx3a$ and $hmx3b$, but only one $hmx1$, $hmx2$, and $hmx4$ gene.

Previous research has shown that Hmx2 and Hmx3 have crucial functions in ear development in mouse, and our recent work shows that this is also the case for Hmx3a in zebrafish (Wang et al. 1998; Wang et al. 2001; Wang et al. 2004; Wang and Luarkin 2005; Hartwell et al. 2019). In mouse, both Hmx2 and Hmx3 mutants have ear defects and these are more severe in double mutants (Wang et al. 2001; Wang et al. 2004). Hmx2 and Hmx3 are also required for correct specification of the mouse hypothalamus (Wang et al. 2004), and morpholino knockdown experiments have suggested that they are required for correct lateral line development in zebrafish (Feng and Xu 2010). Hmx2 and Hmx3 are also expressed in two distinct domains in mouse spinal cord, but the spinal cord functions of these genes are unknown (Bober et al. 1994; Wang et al. 2000; Wang et al. 2004).

Here, we show that zebrafish $hmx2$ and $hmx3a$ are coexpressed in spinal d12 and V1 interneurons, whereas $hmx3b$, $hmx1$, and $hmx4$ are not expressed in spinal cord. Using knockdown and mutational analyses, we demonstrate that, in addition to its role in ear development, $hmx3a$ is required for correct specification of a subset of spinal cord interneuron neurotransmitter phenotypes as well as lateral line progression and viability (survival to adulthood). Our data suggest that in the absence of functional Hmx3a protein, a subset of d12 spinal interneurons switch their neurotransmitter phenotype from glutamatergic (excitatory) to GABAergic (inhibitory). This is important because currently very little is known about how d12 spinal interneuron neurotransmitter phenotypes are specified, or indeed, how spinal cord excitatory neurotransmitter phenotypes in general are specified, and if neurons do not acquire the correct neurotransmitter phenotypes, they cannot function appropriately in spinal cord circuitry.

Surprisingly, despite the fact that $hmx2$ and $hmx3a$ have similar expression patterns during embryonic development and both genes are required for correct ear development in mouse, our mutational analyses did not uncover any requirement for $hmx2$, by itself, in viability, ear development, lateral line progression, or specification of spinal cord interneuron neurotransmitter phenotypes in zebrafish. This is surprising, especially given that embryos injected with a $hmx2$ morpholino have reduced numbers of spinal cord glutamatergic neurons and a corresponding increase in the number of inhibitory spinal cord neurons, and that embryos injected with both $hmx2$ and $hmx3a$ morpholinos have more severe spinal cord phenotypes than single knockdown (SKD) embryos. Zebrafish $hmx2$ mutants are viable and have no obviously abnormal phenotypes in these sensory structures and neurons that require $hmx3a$, even when almost all of the $hmx2$ locus is deleted. (In our most severe mutant allele, $hmx2^{SU39}$, only 84 nucleotides of 5’ and 60 nucleotides of 3’ coding sequence remain). In addition, zebrafish embryos homozygous for deletions of both $hmx2$ and $hmx3a$ have identical phenotypes to severe $hmx3a$ single mutants. However, mutating $hmx2$ in hypomorphic $hmx3a^{SU42}$ mutants, that usually develop normally, results in abnormal ear and lateral line progression phenotypes, suggesting that while $hmx2$ cannot compensate for mutations in $hmx3a$, it does function in these developmental processes, although to a much lesser extent than $hmx3a$. Our analyses of homozygous mutant phenotypes for several different $hmx3a$ mutant alleles also suggest that Hmx3a may not require its homeodomain for its roles in viability or embryonic development. This is surprising, as homeodomain proteins usually function by binding DNA through their homeodomain and regulating gene expression. In contrast, our mutational analyses suggest that Hmx3a may only require its N-terminal domain for its vital functions in viability and sensory organ and spinal cord interneuron development.

Materials and Methods

Ethics statement

All zebrafish experiments in this research were carried out in accordance with the recommendations and approval of either the UK Home Office or the Syracuse University Institutional Animal Care and Use Committee.

Zebrafish husbandry and fish lines

Zebrafish (Danio rerio) were maintained on a 14-hr light/10-hr dark cycle at 28.5°C. Embryos were obtained from natural paired and/or grouped spawns of wild-type (WT; AB, TL, or AB/TL hybrid) fish; heterozygous or homozygous $hmx2$, $hmx3a$, or $hmx2;3a$ mutants (created as part of this study and reported here); $\text{Tg(eox1:EGFP)}^{SU1}$ or $\text{Tg(eox1:EGFP)}^{SU2}$ transgenic fish (Juárez-Morales et al. 2016); $\text{Tg(UAS:mRFP)}$ transgenic fish (Balcuieniene et al. 2013); heterozygous $\text{mindbomb1 (mib1tm528)}$ mutants (Jiang et al. 1996); and heterozygous or homozygous $hmx3a^{tm2954}$ mutants (Kettleborough et al. 2013).

CRISPR mutagenesis and screening

The $hmx3a^{SU3}$ allele was described previously (Hartwell et al. 2019). With the exception of $hmx3a^{tm2954}$ (generated in the Zebrafish Mutation Project and obtained from the Zebrafish International Resource Centre), we created all of the other $hmx2$, $hmx3a$, and $hmx2;3a$ double deletion mutants described in this paper using CRISPR mutagenesis. For all alleles, other than the $hmx2^{MENThU}$ allele, we designed and synthesized single guide RNA (sgRNA) and Cas9 messenger RNA (mRNA) as in Hartwell et al. (2019). For the $hmx2^{MENThU}$ allele, we designed the CRISPR RNA (crRNA) using the Microhomology-mediated End joining kNockout Target Heuristic Utility (MENThU) tool (version 2.1.2), in
the Gene Sculpt Suite (Ata et al. 2018; Mann et al. 2019). The MENTHU allele  crRNA design was verified with CHOPCHOP (version 3.0.0) (Montague et al. 2014; Labun et al. 2016; Labun et al. 2019) and the CRISPR-Cas9 guide RNA design checker tool (Integrated DNA Technologies). The hmx2^MENTHU^ crRNA was purchased together with a universal 67mer trans-activating CRISPR RNA (tracrRNA) (1072533) and Alt-R S.p. Cas9 Nuclease V3 (1081058) from Integrated DNA Technologies. See Table S2 for guide RNA sequences and Figure 4 for their genomic loci. hmx2^SU35^, hmx2^SU36^, hmx2^SU37^, and hmx3a^SU42^ alleles were all generated with a single sgRNA (sgRNA E for hmx2^SU35^, hmx2^SU36^, and hmx2^SU37^; sgRNA F for hmx3a^SU42^; Table S2; Figure 4). hmx2^MENTHU^ was generated with a single crRNA (sgRNA D; Table S2; Figure 4). hmx2^SU38^, hmx2^SU39^, hmx2;hmx3a^SU44^, and hmx2;hmx3a^SU45^ alleles are all deletions, generated by combinatorial use of two sgRNAs. The hmx2^SU38^ sgRNAs (sgRNAs E and F; Table S2; Figure 4) flank the homeodomain. For hmx2^SU39^ we used the same 3' sgRNA and a more 5' sgRNA (sgRNAs C and F; Table S2; Figure 4). To make hmx2;hmx3a double deletion alleles, we designed sgRNAs that flanked the two genes, which are adjacent on chromosome 17 (sgRNAs A and F; Table S2; Figure 4). In all cases except the hmx2^MENTHU^ mutant, we injected 2–4 nl of a mixture of 200 ng/μl of each sgRNA + 600 ng/μl nls-ZCas9-nls mRNA into the single cell of a one-cell stage AB WT embryo. To create the hmx2^MENTHU^ mutant allele, we injected 1 nl of a 5 μM crRNA:tracrRNA:Cas9 ribonucleoprotein complex into the single cell of a very early one-cell stage embryo from an incross of heterozygous hmx3a^SU42^ fish. The 5 μM crRNA:tracrRNA:Cas9 ribonucleoprotein complex was synthesized as described in Hoshijima et al. (2019). hmx3a^SU43^ is a hsp70:Gal4 knock-in allele. We co-injected a donor template containing Gal4, under the control of a minimal hsp70 promoter (pMbaht-hsp70:Gal4, a kind gift of Dr Shin-ichi Higashijima; Kimura et al. 2014) with two sgRNA molecules: one specifically targeting hmx3a (sgRNA B; see Table S2 and Figure 4), and one (Mbaht sgRNA, GGCCT CTGGCCGTTCAGAGG) specifically linearizing the donor template in vivo, into the single cell of one-cell stage embryos from an incross of heterozygous Tg(UAS:mRFP) fish. For these experiments, embryos were injected with 2–4 nl of a mixture of 130 ng/μl of each sgRNA + 180 ng/μl nls-ZCas9-nls mRNA + 66 ng/μl pMbaht-hsp70:Gal4 donor DNA. We screened injected embryos for RFP fluorescence in patterns consistent with hmx3a expression (i.e., ear, lateral line primordium, and/or spinal cord) from 1 day postfertilization (d) onward, and raised injected embryos displaying appropriate expression patterns to adulthood. We then assessed germline transmission by outcrossing to heterozygous or homozygous Tg(UAS:mRFP) fish. Gal4 expression in hmx3a^SU43^ recapitulates hmx3a spinal expression but is not expressed in the ear or lateral line primordium (data not shown).

We identified founder fish for hmx2^SU35^, hmx2^SU36^, hmx2^SU37^, and hmx3a^SU42^ alleles using high-resolution melt analysis (HRMA), and the supermix and amplification programs described in Hartwell et al. (2019). For the PCRs described below, we used Phusion High-Fidelity DNA Polymerase (M0530L; New England BioLabs Inc.) unless otherwise stated. HRMA primers and PCR primers for sequencing are provided in Table S2.

We used the following PCR conditions to identify hmx2^SU38^ founder fish: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 67.0° for 30 sec, 72.0° for 40 sec; followed by a final extension at 72.0° for 5 min. We distinguished the mutant allele by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). The WT allele generated a 1098 bp product, compared with a 671 bp mutant allele product. The PCR primer sequences are provided in Table S2.

We used nested PCR to identify hmx2^SU39^ founder fish, with the following conditions: nested PCR 1: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 69.0° for 20 sec, 72.0° for 75 sec; followed by a final extension at 72.0° for 5 min. The mutant allele was distinguished by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). The WT allele generated a 2012 bp product (which may or may not be detected on the gel), compared with a 576 bp mutant allele product. We then diluted the nested 1 PCR product 1:5 in sterile distilled water and performed the nested PCR 2 reaction using the following conditions: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 66.0° for 20 sec, 72.0° for 60 sec; followed by a final extension at 72.0° for 5 min. The mutant allele was distinguished by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). The WT allele generated a 1758 bp product compared with a 322 bp mutant allele product. All PCR primer sequences are provided in Table S2.

We identified hmx2^MENTHU^ F0 embryos by PCR, followed by sequencing with the forward primer that generated the amplicon (Table S2). The PCR was performed on DNA extracted from individual embryos using the following conditions: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 64.0° for 20 sec, 72.0° for 15 sec; followed by a final extension at 72.0° for 5 min. We assayed that the PCR was successful by gel electrophoresis on a 2.5% TBE agarose gel (100V for 40 min). The PCR product generates a 155 bp product. The PCR product was purified using EZ-10 Spin Column PCR Products Purification Kit (BS664; Bio Basic) and eluted in 30 μl sterile water before sequencing.

We used either assessment of germline transmission, as described above, or PCR to identify hmx3a^SU43^ founder fish. PCR conditions were 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 69.0° for 20 sec, 72.0° for 60 sec; followed by a final extension at 72.0° for 5 min. The mutant allele was distinguished by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). A 1471 bp PCR product was only generated when the reverse primer only recognizes the mutant allele by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). The WT allele generated a 1758 bp product compared with a 322 bp mutant allele product. All PCR primer sequences are provided in Table S2.

We identified hmx2;hmx3a^SU44^ and hmx2;hmx3a^SU45^ founder fish by nested PCR, using the following conditions: nested PCR 1: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 67.0° for 20 sec, 72.0° for 30 sec; followed by a final
extension at 72.0° for 5 min. The mutant allele was distinguished by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). The WT product was too large to be generated by these PCR conditions, so only heterozygous animals were detected by the presence of a 514 bp product on the gel. We then diluted the nested 1 PCR 1:5 in sterile distilled water and performed the nested PCR 2 reaction using the following conditions: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 66.0° for 20 sec, 72.0° for 30 sec; followed by a final extension at 72.0° for 5 min. The mutant allele was distinguished by gel electrophoresis on a 1% TAE agarose gel (110V for 30 min). Again, the WT product was too large to be generated by these PCR conditions, so only heterozygous animals were detected by the presence of a 445 bp product.

Once stable lines were established, we identified hmx2\textsuperscript{SU35} fish by PCR, followed by sequencing (the mutation introduces a 1 bp insertion that cannot be resolved by restriction digestion, and we cannot distinguish heterozygotes from homozygotes using HRMA). We performed this PCR using Taq DNA Polymerase (M0320S; New England BioLabs Inc.) and the following conditions: 95.0° for 30 sec; 35 cycles of 98.0° for 20 sec, 52.0° for 30 sec, 68.0° for 45 sec; followed by a final extension at 68.0° for 5 min. The PCR primer sequences are provided in Table S2. We used HRMA and the conditions described above to identify hmx2\textsuperscript{SU36} stable mutants. Homozygous mutants segregate from heterozygous animals by the scale of their deflection in the HRMA plot. We identified hmx2\textsuperscript{SU37} mutants by performing the PCR used to sequence hmx2\textsuperscript{SU35} stable mutants (see above and Table S2). When we analyzed the products on a 1% TAE gel (110V for 30 min), the WT allele generated a 580 bp product, compared with a 528 bp mutant product. We identified hmx2\textsuperscript{SU38} stable mutants using the same PCR conditions initially used to identify founders (see above and Table S2). We used the same nested PCR conditions to identify hmx2\textsuperscript{SU39} mutants. However, the WT product was not always visible on the gel. Therefore, we also performed a WT amplicon PCR identical to that described above for identifying stable hmx2\textsuperscript{SU35} fish, as this genomic region is only present in WT and heterozygous animals (see also Table S2).

We identified stable hmx3a\textsuperscript{SU42} mutants by PCR, using Taq DNA Polymerase (M0320S; New England BioLabs Inc.) and the following conditions: 94.0° for 2 min; 35 cycles of 94.0° for 30 sec, 64.9° for 30 sec, 72.0° for 30 sec; followed by a final extension at 72.0° for 2 min. The PCR primer sequences are provided in Table S2. While the mutant PCR product (321 bp) could sometimes be distinguished from the WT product (331 bp) by running on a 2% TBE gel (100V for 55 min), the mutation also deletes a Banl restriction site. Following digestion with Banl (R0118S; New England BioLabs Inc.), the products were run on a 2% TBE gel (100V for 40 min). The WT amplicon digested to completion, producing 120 bp + 211 bp bands, whereas the mutant product did not cut. We identified stable hmx3a\textsuperscript{SU3} mutants by running the same PCR used to identify hmx3a\textsuperscript{SU42} mutants (see above and Table S2). The insertion in hmx3a\textsuperscript{SU3} was easily visualized on a 2% TBE gel. The WT product was 331 bp, compared to a mutant product of 400 bp. Since the PCR used to detect hmx3a\textsuperscript{SU43} mutants was specific to the inserted donor DNA, and the WT amplicon in hmx2;hmx3a\textsuperscript{SU44} and hmx2;hmx3a\textsuperscript{SU45} mutants was too large to detect using the nested PCR conditions, for these alleles we also performed a WT amplicon PCR to distinguish WTs from heterozygotes. The WT amplicon PCR was identical to that performed before Banl digestion on hmx3a\textsuperscript{SU42} mutants (see above and Table S2). For hmx3a\textsuperscript{SU43}, the WT amplicon PCR results were compared to the PCR results (identical PCR to that first used to identify founders, see above), and for hmx2;hmx3a\textsuperscript{SU44} and hmx2;hmx3a\textsuperscript{SU49}, the WT amplicon PCR results were compared to the nested 2 PCR results (identical nested 2 PCR to that first used to identify founders, see above and Table S2).

In all cases, stable F1 heterozygous fish were confirmed by sequencing. To further confirm the mutant sequences of hmx2\textsuperscript{SU39} and hmx3a\textsuperscript{SU42}, we extracted total RNA from embryos produced by incrosses of homozygous viable adults using TRIzol Reagent (15596018; Thermo Fisher Scientific) and the RNase Mini Kit (74104; QIAGEN, Valencia, CA). Total RNA was converted to complementary DNA (cDNA) using the iScript cDNA synthesis kit (1708891; Bio-Rad, Hercules, CA). We performed transcript-specific PCRs using the following primers and conditions: hmx2-forward: TGAACGTGTATGAGACGAGGAAATGGA and hmx2-reverse: GTGTATTTTGTAACGGTTCTTATGTGTGT (PCR: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 64.2° for 20 sec, 72.0° for 30 sec; followed by final extension at 72.0° for 5 min); or hmx3a-forward: ACCGGCGTCCAATCCATTG and hmx3a-reverse: GTGGAGTTAAACCCGATGAG (PCR: 98.0° for 30 sec; 35 cycles of 98.0° for 10 sec, 71.0° for 20 sec, 72.0° for 30 sec; followed by final extension at 72.0° for 5 min). We then confirmed these homozygous mutant transcript sequences by sequencing.

**Morpholino injections**

For SKD translation-blocking experiments, 3.5 nl of a mixture containing either 2 ng/nl of a translation-blocking hmx2 morpholino (MO) (5’ TTCGCTGTCCCTCAGAATTCCAT) or 2 ng/nl of a translation-blocking hmx3a morpholino (5’ ACCTATCCTGTGTTTTCGGGCAT) plus 5 ng/nl of a control zebrafish p53 morpholino (5’ GCGCCATTGCTTTGCAAAT) was injected into the single cell of a one-cell stage WT embryo. For double knockdown (DKD) experiments with translation-blocking morpholinos, 3.5 nl of a mixture containing 2 ng/nl of both translation-blocking hmx3a morpholinos plus 5 ng/nl of the control zebrafish p53 morpholino was injected. For DKD splice-blocking experiments, 4 nl of a mixture containing 5 ng/nl of both a splice-blocking hmx2 morpholino (5’ GGGAAGGACCAACATGGCAGAC) and a splice-blocking hmx3a morpholino (5’ GGTGTCTCAGGAAATAGGCCAAA), plus 7 ng/nl of the control zebrafish p53 morpholino was injected (all morpholinos obtained from Gene Tools). DKD but not SKD embryos exhibit delayed development from somitogenesis stages onward when...
compared to un.injected controls. To circumvent this, they were incubated at 32°C from 9 hours post fertilization (hpf) onward, whereas the un.injected controls remained at 28.5°C. This ensured that control and injected embryos reached the desired developmental stage of 27 hpf at approximately the same time. The lateral line primordium does not migrate in DKD animals, so this could not be used to stage injected embryos. Instead, these embryos were visually inspected and fixed when they displayed the same head-trunk angle, head size, and eye size as prim-staged un.injected control embryos (Kimmel 1995). Migration of the lateral line primordium is unaffected in SKD embryos, so these were prim-staged before fixing for experiments (Kimmel 1995). Morpholino injections always produce a spectrum of phenotypes, since it is hard to ensure that every cell receives the same dose. Therefore, before fixing at 27 hpf, we removed any embryos with severely abnormal morphology (stunted length and/or severely developmentally delayed, likely caused by receiving too much morpholino). Embryos injected with hmx2/3a morpholinos (SKD and DKD) display a slight curled-tail-down morphology. Embryos that lacked this morphology (and may therefore not have received any sufficient morpholino) were also removed before fixing.

For the mRNA + morpholino rescue experiments, we co-injected either one individual or both translation-blocking hmx morpholinos (at the same volume and dose described above), together with a total dose of up to 500 pg of morpholino-resistant (MOR) full-length hmx2 or hmx3a mRNA. Both hmx mRNAs had seven nucleotides altered in the morpholino recognition sequence. Each change was in the third nucleotide of a codon. This codon wobble was used so that the same amino acid was encoded in each case, but the mRNA would not be recognized by the morpholino. The protein encoded by the injected mRNA is therefore the same as either endogenous Hmx2 or Hmx3a.

WT hmx2: ATG AAT AAT TCG GAG GAC AGC (Met, Asn, Asn, Ser, Glu, Asp, Ser)  
MOR–hmx2: ATG AAC AAC TCC GAA GAT AGT (Met, Asn, Asn, Ser, Glu, Asp, Ser)  
WT hmx3a: ATG CCC GAA ACA ACA CAG GAT AGC (Met, Pro, Glu, Thr, Thr, Gln, Asp, Thr)  
MOR–hmx3a: ATG CCC GAG ACT ACT CAA GAC ACC (Met, Pro, Glu, Thr, Thr, Gln, Asp, Thr)

RT-PCR was performed to assess the efficiency of hmx2;hmx3a DKD by splice-blocking morpholinos (Figure S1, A and C). At 27 hpf, separate pools of 25 injected embryos (injected at the one-cell stage with the morpholino dose and volume described above) and 25 uninjected control embryos were homogenized in 200 μl of Tri Reagent Solution (AM9738; Thermo Fisher Scientific). Total RNA was extracted and purified as per the manufacturer’s instructions, before resuspending in 20 μl of sterile distilled water. To remove genomic DNA, 2.4 μl of RQ1 DNase Buffer and 2 μl of RQ1 RNase-Free DNase (M6101; Promega, Madison, WI) was added to each RNA sample and incubated for 15 min at 37°C. Heat inactivation of the DNase was performed for 10 min at 65°C. 20 μl RT-PCRs were performed as per the manufacturer’s instructions using the Qiagen One-Step RT-PCR kit (210210; Qiagen) and the following primers: hmx2 RT-PCR E1-2 forward: TCAAGTTTCCGATCCAGTCTA and hmx2 RT-PCR E1-2 reverse: ATAAAACGTGACCTCCGAGA GAA; hmx3a RT-PCR E1-2 forward: GTCAAGCTTAAGCC TATTITG and hmx3a RT-PCR E1-2 reverse: TCATC TTCTCCAGTCGCTA; and actb1 RT-PCR E3-4 forward: GAGGTATCGTCTGGTCAGGTC (universal PCR program: 50°C for 30 min; 95°C for 15 min; 35 cycles of 95°C for 30 sec, 57°C for 45 sec, and 72°C for 1 min; followed by a final extension for 10 min at 72°C). Parallel reactions, omitting reverse transcriptase and on non-DNase-treated samples, were used to verify the nonspliced (genomic) PCR product. 10 μl of each RT-PCR product was assessed by electrophoresing for 40 min at 100 V on a 2% TBE agarose gel. The hmx2 RT-PCR E1-2 primers generate either a 1204 bp genomic (unspliced) or 426 bp spliced product. The hmx3a RT-PCR E1-2 primers generate either a 779 bp genomic (unspliced) or 393 bp spliced product. The actb1 RT-PCR E3-4 primers generate either a 697 bp genomic (unspliced) or 387 bp spliced product (Figure S1, A–D).

To assess whether genetic compensation occurs in either hmx2SU39 or hmx3aSU42 mutants, which lack obvious phenotypes, or hmx3aSU3 mutants, which have milder spinal cord phenotypes than hmx2;hmx3a DKD embryos, we injected the same dose of either hmx2 + p53 MOs (hmx2SU39) or hmx3a + p53 MOs (hmx3aSU42, hmx3aSU3) as described above, into the single cell of one-cell stage embryos generated from incrosses of heterozygous hmx2SU39, hmx3aSU42, or hmx3aSU3 parents, respectively. If genetic compensation is occurring, the upregulated compensating gene(s) will not be knocked down by the hmx morpholino and the phenotype of homozygous mutants should be unchanged. In contrast, WT and heterozygous animals, which contain at least one WT copy of the respective hmx gene will be susceptible to the hmx morpholino and should exhibit stronger, morphant-like phenotypes. For these experiments, while we removed any embryos with severely abnormal morphology, we did not remove embryos that lacked the curled-tail-down morphology, in case these were morpholino-resistant mutant embryos. After fixing, we performed an in situ hybridization for the glutamatergic marker, slc17a6a/b. We visually inspected the embryos on a dissecting microscope and categorized them as either the stronger, morphant-like phenotype (large reduction in the number of slc17a6a/b-expressing cells) or a more subtle phenotype (WT-like in the case of hmx2SU39 and hmx3aSU42, or a smaller reduction in the number of slc17a6a/b-expressing cells in the case of hmx3aSU3 embryos). Embryos within each class were then genotyped as described in the CRISPR mutagenesis section above.
Genotyping

We isolated DNA for genotyping from both anesthetized adult fish and fixed embryos via fin biopsy or head dissections, respectively. For assaying ear phenotypes, we dissected tail tips instead. We genotyped the hmx CRISPR mutants as described above. For mib1a52b and hmx3a23054 mutants, we used KASP assays designed by LGC Biosearch Technologies. KASP assays use allele-specific PCR primers, which differentially bind the fluorescent dyes that we quantified with a Bio-Rad CFX96 real-time PCR machine to distinguish genotypes. The proprietary primers used were mib_tas52b and hmx3a_sas23054. Heads or tail tips of fixed embryos were dissected in 70% glycerol/30% distilled water with insect pins. Embryo trunks were stored in 70% glycerol/30% distilled water at 4°C for later analysis. For all experiments except phalloidin-staining experiments, DNA was extracted via the HotSHOT method (Truett et al. 2000) using 10 μl of 50 mM NaOH and 1 μl of 1M Tris-HCl (pH 7.4). For phalloidin-staining experiments, the tail up until the end of the yolk extension was dissected in 70% glycerol/30% distilled water as described above and transferred to PBS with 0.1% Tween-20 (PBST). The PBST was then replaced with 50 μl of DNA extraction buffer [10 mM Tris, pH 8.0, 10 mM EDTA, 200 mM NaCl, 0.5% SDS, 200 μg/ml Proteinase K (Proteinase K, recombinant, PCR grade, 3115879001; Sigma Aldrich, St. Louis, MO), before incubating for 3 hours in a 55°C water bath. The samples were vortexed periodically to ensure thorough digestion of the tissue. Subsequently, the Proteinase K was inactivated by heating the samples for 10 min at 100°C, before centrifuging for 20 min at 14,000 rpm at room temperature to pellet debris. The supernatant was transferred to sterile microcentrifuge tubes before adding 20 μg UltraPure Glycogen (10814010; Thermo Fisher Scientific) and 2 volumes of ice-cold RNase-free ethanol. Samples were precipitated at −20°C overnight. Genomic DNA was recovered by centrifugation at 4°C, followed by washing with 70% RNase-free ethanol and further centrifugation at 4°C. After carefully removing the ethanolic supernatant, the pellets were air dried for 5–10 min at room temperature before resuspending in 15 μl of sterile distilled water.

In situ hybridization and immunohistochemistry

We fixed embryos in 4% paraformaldehyde/PBS and performed single and double in situ hybridizations and immunohistochemistry plus in situ hybridization double-labeling experiments as previously described (Concordet et al. 1996; Batista et al. 2008). Sources of in situ hybridization probes are provided in Table S1. To amplify in situ hybridization probe templates for hmx1 and hmx3b, we created cDNA from 27 hpf WT zebrafish embryos. We extracted total RNA by homogenizing 50–100 mg of embryos in 1 ml of TRIzol reagent (15596-026; Ambion). We confirmed RNA integrity (2:1 ratio of 28S:18S ribosomal RNA bands) and quality (A260/A280 ratio of ∼2.0) using agarose gel electrophoresis and spectrophotometry, respectively. We synthesized cDNA using Bio-Rad iScript Reverse Transcription Supermix kit (170-8891; Bio-Rad). We amplified hmx1 sequence from the cDNA using Phusion High-Fidelity DNA Polymerase (M0530L; New England BioLabs Inc.), primers hmx1-forward: CTGGTATATT TGTCGAACACATGC and hmx1-reverse: GCTTCCTGCTGA CACAGTTCG, and PCR conditions 98.0°C for 10 sec; 30 cycles of 98.0°C for 60 sec, 57.0°C for 30 sec, and 72.0°C for 30 sec; followed by a final extension for 45 sec at 72.0°C. The PCR product was assessed on a 1% TAE gel, before purifying with Qiagen PCR Purification Kit (28104; Qiagen). We used Taq DNA polymerase (M0320S; New England BioLabs Inc.) to add 3′A overhangs before TOPO TA-cloning (K4600-01; Invitrogen, Carlsbad, CA). We then performed colony PCR using the same PCR primers and conditions used to amplify the hmx1 sequence from cDNA. We extracted plasmid DNA from positive colonies using Qiagen Spin Miniprep Kit (27104; Qiagen) and then verified the sequence using standard Sanger sequencing. To make the antisense RNA riboprobe, we linearized DNA with HindIII-HF (R3104S; New England BioLabs Inc.) and transcribed with T7 RNA Polymerase (10881767001; Roche). We used a PCR-based DNA template to make the hmx3b ISH probe. The reverse primer contains the T3 RNA Polymerase minimal promoter sequence (underlined). We used primers hmx3b-forward: GTGTGCCCCGTCACTTACCAC and hmx3b-reverse: AATTAACCCCTACTAAAGGGATGAATGATGAAGATGCG CAAC, 27 hpf WT cDNA, Phusion High-Fidelity DNA Polymerase (M0530L, New England BioLabs Inc.) and PCR conditions: 94.0°C for 3 min; 35 cycles of 94.0°C for 30 sec, 56.5°C for 30 sec, and 72.0°C for 1.5 min; followed by a final extension step of 72.0°C for 10 min. We purified the template through phenol:chloroform:isoamyl alcohol extraction and precipitation with 0.2 M NaCl and ice-cold ethanol before in situ probe synthesis using 1 μg purified PCR product, T3 RNA Polymerase (11031171001; Roche), and DIG RNA Labeling Mix (11277073910; Roche).

Embryos older than 24 hpf were usually incubated in 0.003% 1-phenyl-2-thiourea to prevent pigment formation. For some experiments (indicated in the results) we added 5% dextran sulfate to the hybridization buffer. Dextran sulfate can increase specific staining in in situ hybridization experiments as it facilitates molecular crowding (Ku et al. 2004; Lautar et al. 2011).

In cases where we did not detect expression of a particular gene in the spinal cord, we checked for low levels of expression by exposing embryos to prolonged staining. In some cases, this produced higher background (diffuse, nonspecific staining), especially in the hindbrain, where ventricles can sometimes trap antisense riboprobes.

To determine neurotransmitter phenotypes, we used probes for genes that encode proteins that transport or synthesize specific neurotransmitters, as these are some of the most specific molecular markers of these cell fates [Higashijima et al. (2004b,c) and references therein]. A mixture of probes to slc17a6a and slc17a6b (previously called vglut), which encode glutamate transporters, was used to label glutamatergic neurons.
neurons (Higashijima et al. 2004b,c). GABAergic neurons were labeled using probes to gad1b (probes previously called gad67a and gad67b) (Higashijima et al. 2004b,c). The gad1b gene encodes for a glutamic acid decarboxylase, which is necessary for the synthesis of GABA from glutamate. A mixture of probes (glyt2a and glyt2b) for slc6a5 (previously called glyt2) was used to label glycineergic cells (Higashijima et al. 2004b,c). slc6a5 encodes for a glycine transporter necessary for glycine reuptake and transport across the plasma membrane.

The antibodies that we used for fluorescence in situ hybridization were mouse anti-Dig (200-002-156; 1:5000; Jackson ImmunoResearch) and rabbit anti-Flu (A889; 1:2500; Invitrogen). These were detected using secondary antibodies goat anti-rabbit-HRP (G-21324; 1:750; Thermo Fisher Scientific) and goat anti-mouse-HRP (G-21040; 1:750; Thermo Fisher Scientific), and Tyramide SuperBoost Kits B40922 and B40915 (Thermo Fisher Scientific).

For double-fluorescence in situ hybridization and immunohistochemistry, after detection of the in situ hybridization reaction using Tyramide SuperBoost Kit B40915 (with HRP, goat anti-mouse IgG and Alexa Fluor 594 Tyramide), embryos were washed eight times for 15 min in PBST and incubated in Image-iT FX Signal Enhancer (I36933; Thermo Fisher Scientific) for 30 min at room temperature. Immunohistochemistry was performed using chicken polyclonal anti-GFP primary antibody (Ab13970; 1:500; Abcam) and a goat anti-chicken IgY (H+L), Alexa Fluor 488 secondary antibody (A-11039; 1:1000; Thermo Fisher Scientific).

**Phalloidin staining**

Four-day-old embryos generated from crosses of heterozygous hmx2Su39 or hmx2;hmx3aSu44 parents were fixed and processed for phalloidin staining as described in Hartwell et al. (2019). Stained embryos were stored in DABCO [2% w/v 1,4-Diazobicyclo[2.2.2]octane (D27802; Sigma Aldrich) in 80% glycerol in sterile distilled water).

**Quantitative PCR analyses**

We collected embryos from crosses of AB WT parents and flash-froze them at 16-cell, 6, 14, 27, and 48 hpf stages. We collected 40–50 embryos per biological replicate per developmental stage and performed duplicate biological replicates. We isolated total RNA by homogenizing each sample in 1 ml of TRIzol reagent (15596-026; Ambion). Following chloroform extraction, we added 20 μg UltraPure Glycogen (10814010; Thermo Fisher Scientific) to the aqueous phase followed by one volume of RNase-free ethanol. We performed RNA purification and genomic DNA removal using the Monarch Total RNA Miniprep Kit (T2010S; New England BioLabs Inc.), following manufacturer’s instructions for purifying TRIzol-extracted samples. RNA concentration was measured using Nanodrop 2000 (ND2000; Thermo Fisher Scientific), before synthesizing cDNA using the Bio-Rad iScript Reverse Transcription Supermix kit (170-8891; Bio-Rad). We also included controls lacking reverse-transcriptase to assay for the presence of genomic DNA contamination. Quantitative PCR (qPCR) was performed in triplicates for each sample using iTaq Universal SYBR Green Supermix (1725121; Bio-Rad) and a Bio-Rad CFX96 real-time PCR machine. The following qPCR primers were used: hmx2-qPCR-forward: CCCCATTTCAGTTTTCAGATCCGTC and hmx2-qPCR-reverse: TGCTTCCTTTTGTAATCCGTTAG; hmx3a-qPCR-forward: TTATGGCAGCTTCCCTTTTC and hmx3a-qPCR-reverse: ACTGTTCCTCCAGTCGCTATGC; and mob4-qPCR-forward: CACCCGTTTCGATGTAAGTACA and mob4-qPCR-reverse: GTTAAGCGAGATTACAAATGGAG.

The hmx2 and hmx3a primers were generated in this study. The mob4 primers were generated by Hu et al. (2016). They demonstrated that mob4 is a more effective reference gene than actb2 across a broad range of zebrafish developmental stages, including early stages where maternal mRNAs should be present (Hu et al. 2016). To generate amplification data the program used was 95.0°C for 30 sec; 40 cycles of 95.0°C for 5 sec and 63.3°C (hmx2)/64.5°C (hmx3a)/60.0°C (mob4) for 30 sec; with imaging after each cycle. To assay amplification specificity and exclude false positives from primer dimers we then generated melt data using 65.0°C for 30 sec; 40 cycles of 65.0°C–95.0°C, +0.5°C/second increment, with each increment held for 5 sec before imaging; 95.0°C for 15 sec.

**Screening lateral line and otolith phenotypes**

We examined whether any of the hmx mutants generated in this study had lateral line and/or fused otolith phenotypes, as reported for hmx2;hmx3a DKD embryos (Feng and Xu 2010). To assay live lateral line phenotypes, we anesthetized embryos from crosses of heterozygous mutant fish in 0.16% tricaine (A5040; Sigma Aldrich) in embryo medium [5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2.2H2O, 0.33 mM MgSO4.7H2O, 0.017% w/v (0.7 mM) HEPES pH 7.8 and 0.00004% methylene blue in autoclaved reverse osmosis water] and mounted them on coverslip bridges [2 × 22 mm square glass coverslips (16004-094; VWR) glued together on either side of a 24 × 60 mm glass cover slip (124605; Thermo Fisher Scientific), overlaid with a third 22 mm square glass coverslip]. Using a Zeiss Axio Imager M1 compound microscope, we located the tip of the lateral line primordium and counted the somite number adjacent to this position. We also used this method routinely to determine the developmental stage of embryos before fixing for in situ hybridization. To assay lateral line phenotypes in fixed embryos, we performed in situ hybridizations for hmx3a or krt15 (both of which label the migrating primordium and neuramostas) and then determined the lateral line position as in live embryos. To examine live otolith phenotypes, embryos were raised until 3 d, before anesthetizing (as for assessing live lateral line phenotypes) and examining the spatial location of otoliths in both ears. WT embryos have two otoliths in each ear: one smaller, anterior (utricular) otolith, and one larger, posterior (saccular) otolith. These are separate from each other and spatially distinct. We classified otoliths as fused if only one...
large, amalgamated otolith was visible in a midventral position within the otic vesicle.

**Imaging**

Embryos were mounted in 70% glycerol:30% distilled water and differential interference contrast (DIC) pictures were taken using an AxioCam MRc5 camera mounted on a Zeiss Axio Imager M1 compound microscope. Fluorescence images were taken on a Zeiss LSM 710 confocal microscope. Images were processed using Adobe Photoshop software (Adobe, Inc.) and Image J software (Abramoff et al. 2004). In some cases, different focal planes were merged to show labeled cells at different medio-lateral positions in the spinal cord. All images were processed for brightness, contrast and color balance using Adobe Photoshop software (Adobe, Inc.). Images of control and mutant embryos from the same experiment were processed identically. Figures were assembled using Adobe Photoshop and Adobe Illustrator (Adobe, Inc.).

**Cell counts and statistics**

In all cases except where noted to the contrary, cell counts are for both sides of a 5-somite length of 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. Embryos from mutant crosses were counted blind to genotype. 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 medio-lateral positions (both sides of the spinal cord; Batista et al. 2008; Batista and Lewis 2008; England et al. 2011; Hilinski et al. 2016; Juárez-Morales et al. 2016).

In some cases, cell count data were pooled from different experiments. Before pooling, all pairwise combinations of data sets were tested to determine if there were any statistically significant differences between them, as described below. Data were only pooled if none of the pairwise comparisons were statistically significantly different from each other. In addition, as in situ hybridization staining can vary slightly between experiments, we only compared different mutant results when the counts from their corresponding WT sibling embryos were not statistically significantly different from each other.

To determine whether differences in values are statistically significant, data were first analyzed for normality using the Shapiro–Wilk test. Data sets with nonnormal distributions were subsequently analyzed using the Wilcoxon–Mann–Whitney test (also called the Mann–Whitney U-test). For data sets with normal distributions, the F-test for equal variances was performed, before conducting either a type 2 (for equal variances) or type 3 (for unequal variances) Student’s t-test. P-values generated by Wilcoxon–Mann–Whitney, type 2 Student’s t-test and type 3 student’s t-test are indicated by †, ‡ and §, respectively. To control for type 1 errors, when comparing three or more experimental conditions, a one-way ANOVA test was performed. Before conducting ANOVA tests, data were first analyzed for normality using the Shapiro–Wilk test, as described above. All data sets for ANOVA analysis had normal distributions and so were subsequently assessed for homogeneity of variances using Bartlett’s test. All of the data sets also had homogeneous (homoscedastic, Bartlett’s test P > 0.05) variances and so standard ANOVA analysis was performed. ANOVA results are reported as F(df1,df2) = F-ratio, P-value = x, where F is the F-statistic, df1 is the degree of freedom for the numerator of the F-ratio, df2 is the degree of freedom for the denominator of the F-ratio, and x is the P-value. For statistically significant ANOVA, to determine which specific experimental groups or groups differed, post hoc testing was performed. Since all ANOVA data sets had homogeneous (homoscedastic) variances, Tukey’s honestly significant difference post hoc test for multiple comparisons was performed. P-values generated by Tukey’s honestly significant difference test are indicated by §. Data are depicted as individual value plots and the n-values for each experimental group are also shown. For each plot, the wider red horizontal bar depicts the mean and the red vertical bar depicts the SEM (SEM values are listed in Tables 1 and 2). Individual data value plots were generated using Prism version 8.4.3 (GraphPad Software, San Diego, California; www.graphpad.com). To assess whether mutant phenotypes occurred at Mendelian frequencies, we performed chi-squared tests. To test whether a small number of embryos with abnormal phenotypes was statistically significantly different from zero we performed a binomial distribution test, using the cumulative distribution function, the number of embryos without mutant phenotypes, the total number of embryos examined (n) and a probability argument of n − 1/n. P-values > 0.05 support the null hypothesis that the number of embryos with abnormal phenotypes is not statistically significantly different from zero. Shapiro–Wilk and Wilcoxon–Mann–Whitney testing was performed in R version 3.5.1 (R Development Core Team 2005). The F-test, Student’s t-test, chi-squared test, and binomial distribution test were performed in Microsoft Excel version 16.41. Bartlett’s testing, standard ANOVA, and Tukey’s honestly significant difference testing were performed in Prism version 8.4.3 (GraphPad Software).

**Microarray expression profiling experiments**

These experiments are described in detail in (Cerda et al. 2009). P values were corrected for multiple testing (Benjamini and Hochberg 1995; Gentleman et al. 2004; Tarraga et al. 2008). These data have been deposited in the NCBI Gene Expression Omnibus with accession number GSE145916.
Table 1 Statistical comparisons of numbers of cells expressing particular genes in morpholino knockdown experiments

| Fig. | Comparison                                                                 | Gene                  | Difference between two means | P-value |
|------|---------------------------------------------------------------------------|-----------------------|------------------------------|---------|
| 3G   | Uninjected control (73.1 ± 0.9) vs. hmx2;hmx3a DKD (71.8 ± 1.3)            | hmx3a                 | 1                             | 0.328   |
| 3J   | Uninjected control (47.8 ± 0.4) vs. hmx2;hmx3a DKD (47.2 ± 1.0)            | en1b                  | 1                             | 0.580   |
| 3M   | Uninjected control (106.1 ± 0.8) vs. hmx2 SKD (89.1 ± 1.5)                 | slc17a6a/b            | 17                            | <0.001  |
| 3M   | Uninjected control (106.1 ± 0.8) vs. hmx3a DKD (84.7 ± 1.3)                | slc17a6a/b            | 21                            | <0.001  |
| 3M   | Uninjected control (106.1 ± 0.8) vs. hmx2;hmx3a DKD (72.2 ± 1.1)           | slc17a6a/b            | 34                            | <0.001  |
| 3M   | hmx2 SKD (89.1 ± 1.5) vs. hmx2;hmx3a DKD (72.2 ± 1.1)                      | slc17a6a/b            | 17                            | <0.001  |
| 3M   | hmx3a SKD (84.7 ± 1.3) vs. hmx2;hmx3a DKD (72.2 ± 1.1)                      | slc17a6a/b            | 13                            | <0.001  |
| 3M   | hmx2 SKD (89.1 ± 1.5) vs. hmx3a SKD (84.7 ± 1.3)                            | slc17a6a/b            | 4                             | 0.186   |
| 3P   | Uninjected control (160.6 ± 0.9) vs. hmx2 SKD (172.0 ± 1.9)                | slc32a1               | 11†                           | <0.001  |
| 3P   | Uninjected control (160.6 ± 0.9) vs. hmx3a SKD (174.6 ± 1.6)               | slc32a1               | 14†                           | <0.001  |
| 3P   | Uninjected control (160.6 ± 0.9) vs. hmx2;hmx3a DKD (190.8 ± 1.6)          | slc32a1               | 30†                           | <0.001  |
| 3P   | hmx2 SKD (172.0 ± 1.92) vs. hmx2;hmx3a DKD (190.8 ± 1.6)                    | slc32a1               | 19†                           | <0.001  |
| 3P   | hmx3a SKD (174.6 ± 1.6) vs. hmx2;hmx3a DKD (190.8 ± 1.6)                    | slc32a1               | 16†                           | <0.001  |
| 3P   | hmx2 SKD (172.0 ± 1.9) vs. hmx3a SKD (174.6 ± 1.6)                          | slc32a1               | 31                            | 0.632   |
| 3S   | Uninjected control (106.1 ± 0.8) vs. hmx2;hmx3a DKD + MOR-hmx2 mRNA (96.2 ± 1.5) | slc17a6a/b            | 10†                           | <0.001  |
| 3S   | Uninjected control (106.1 ± 0.8) vs. hmx2;hmx3a DKD + MOR-hmx3a mRNA (91.5 ± 1.6) | slc17a6a/b            | 15†                           | <0.001  |
| 3S   | hmx2;hmx3a DKD (72.2 ± 1.1) vs. hmx2;hmx3a DKD + MOR-hmx2 mRNA (96.2 ± 1.5) | slc17a6a/b            | 24†                           | <0.001  |
| 3S   | hmx2;hmx3a DKD (72.2 ± 1.1) vs. hmx2;hmx3a DKD + MOR-hmx3a mRNA (91.5 ± 1.6) | slc17a6a/b            | 19†                           | <0.001  |
| 3S   | hmx2;hmx3a DKD + MOR-hmx2 mRNA (96.2 ± 1.5) vs. hmx2;hmx3a DKD + MOR-hmx3a mRNA (91.5 ± 1.6) | slc17a6a/b            | 5†                            | 0.286   |
| 3V   | Uninjected control (106.1 ± 0.8) vs. hmx2 SKD + MOR-hmx2 mRNA (105.6 ± 1.7) | slc17a6a/b            | 1†                            | 0.997   |
| 3V   | Uninjected control (106.1 ± 0.8) vs. hmx2 SKD + MOR-hmx3a mRNA (105.6 ± 1.8) | slc17a6a/b            | 0                             | 0.997   |
| 3V   | hmx2 SKD (89.1 ± 1.5) vs. hmx2 SKD + MOR-hmx2 mRNA (105.6 ± 1.7)            | slc17a6a/b            | 17†                           | <0.001  |
| 3V   | hmx2 SKD (89.1 ± 1.5) vs. hmx2 SKD + MOR-hmx3a mRNA (105.6 ± 1.8)           | slc17a6a/b            | 17†                           | <0.001  |
| 3V   | hmx2 SKD + MOR-hmx2 mRNA (105.6 ± 1.7) vs. hmx2 SKD + MOR-hmx3a mRNA (105.6 ± 1.8) | slc17a6a/b            | 0                             | 1†       |
| 3Y   | Uninjected control (106.1 ± 0.8) vs. hmx3a SKD + MOR-hmx2 mRNA (95.0 ± 1.5) | slc17a6a/b            | 11†                           | <0.001  |
| 3Y   | Uninjected control (106.1 ± 0.8) vs. hmx3a SKD + MOR-hmx3a mRNA (102.4 ± 1.5) | slc17a6a/b            | 4†                            | 0.345   |
| 3Y   | hmx3a SKD (84.7 ± 1.3) vs. hmx3a SKD + MOR-hmx2 mRNA (95.0 ± 1.5)           | slc17a6a/b            | 10†                           | <0.001  |
| 3Y   | hmx3a SKD (84.7 ± 1.3) vs. hmx3a SKD + MOR-hmx3a mRNA (102.4 ± 1.5)          | slc17a6a/b            | 18†                           | <0.001  |
| 3Y   | hmx3a SKD + MOR-hmx2 mRNA (95.0 ± 1.5) vs. hmx3a SKD + MOR-hmx3a mRNA (102.4 ± 1.5) | slc17a6a/b            | 7†                            | 0.063   |

Statistical comparisons between uninjected WT control and knockdown embryos. First column indicates the figure panel that contains the relevant individual value plots for the comparison. Second column states the genotypes being compared. Numbers within parentheses indicate mean numbers of cells ± SEM. In all cases, numbers are an average of at least five embryos and cells were counted in all dorsal-ventral spiral cord rows. All of the experiments were conducted on 27 hpf embryos. 27 hpf embryos fixed on different days varied slightly in stage from prim-stage 9-12. This explains the small differences in numbers of cells labeled with a particular probe in uninjected WT control embryos in different experiments. Data from different days were only combined if there was no statistically significant difference between uninjected WT control embryos for each day (see Materials and Methods). Column three lists the gene that the cell counts and statistical comparison refer to. The fourth column indicates the difference between the two mean values for the embryos being compared. All values are rounded to the nearest whole number. Last column shows the P-value for the comparison, rounded to three decimal places. Statistically significant (P < 0.05) values are indicated in bold. Statistical test used is indicated by superscript symbol: Wilcoxon-Mann-Whitney test (″), type 2 Student’s t-test (‡), and Tukey’s honestly significant post hoc test after ANOVA (*). For a discussion of why particular tests were used, see Materials and Methods. †, increase; ‡, decrease.

Data and reagent availability

Plasmids and zebrafish strains are available upon request. Supplemental material available at figshare: https://doi.org/10.25386.genetics.13108325. Figure S1 contains RT-PCR and cell-count data demonstrating the efficacy of hmx2;hmx3a DKD with splice-blocking morpholinos. Figure S2 shows an alignment of mouse and zebrafish Hmx2 and Hmx3(a) protein sequences. Table S1 includes gene names, ZFIN identifiers, and references for in situ hybridization probes. Table S2 contains the sgRNA and primer sequences used for hmx2,
Results

**hmx2 and hmx3a are the only hmx genes expressed in the spinal cord**

While the expression and functions of zebrafish hmx genes have been analyzed during the development of sensory structures such as the eye and the ear, the expression of hmx1, hmx2, hmx3a, and hmx4 in the developing spinal cord has not been investigated and no expression data has previously been reported for hmx3b, which only appeared in more recent versions of the zebrafish genome sequence (Zv9 and above). Therefore, to determine which of the hmx genes are expressed in the spinal cord we performed *in situ* hybridizations for hmx1, hmx2, hmx3a, hmx3b, and hmx4 at different developmental stages (Figure 1). At all of these stages, we observed no spinal cord expression of hmx1, hmx3b, or hmx4 (Figure 1). However, consistent with previous reports, both hmx1 and hmx4 were expressed in the developing eye, ear, and anterior lateral line neuromasts (Figure 1; French et al. 2007; Feng and Xu 2010; Gonal et al. 2011; Boisset and Schorderet 2012; Marcelli et al. 2014). In contrast, the only expression of hmx3b that we observed was weak hindbrain expression at later stages of development (36–48 hpf; Figure 1, S’, S”, and AC”).

In contrast, hmx2 and hmx3a are expressed in the spinal cord at all of the stages that we examined (Figure 1). The spinal cord expression patterns of these two genes are very similar, with the exception that initially, hmx2 expression appears to be weaker than hmx3a and it does not extend as

### Table 2 Statistical comparisons of numbers of cells expressing particular genes in mutant experiments

| Fig. | Comparison                      | Gene     | Difference between two means | P-value |
|------|---------------------------------|----------|------------------------------|---------|
| NS   | WT (105.5 ± 2.2) vs. hmx2SU47  | scl17a6a/b | 2 | 0.582* |
| NS   | WT (102.8 ± 0.9) vs. hmx2SU48  | scl17a6a/b | 1 | 0.700* |
| 5K   | WT (96.4 ± 1.3) vs. hmx2SU39   | scl17a6a/b | 2 | 0.351* |
| 5G   | WT (106.0 ± 0.9) vs. hmx3aSU2 (100.9 ± 1.1) | scl17a6a/b | 2 | 0.673* |
| 5H   | WT (102.2 ± 1.1) vs. hmx2SU46  | scl17a6a/b | 1 | 0.567* |
| NS   | WT (97.0 ± 1.2) vs. hmx3aSU4 (85.2 ± 1.2) | scl17a6a/b | 12 | 0.019 |
| 5J   | WT (99.8 ± 1.9) vs. hmx3aSU4 (86.4 ± 0.8) | scl17a6a/b | 13 | <0.001* |
| NS   | WT (102.0 ± 1.7) vs. hmx2SU49  | scl17a6a/b | 14 | <0.001* |
| 5I   | WT (102.2 ± 1.9) vs. hmx2/hmx3SU2 | scl17a6a/b | 13 | 0.001* |
| NS   | WT (102.0 ± 1.7) vs. hmx2/hmx3SU3 | scl17a6a/b | 14 | 0.001* |
| 5I and J | hmx3aSU5 (86.4 ± 0.8) vs. hmx2/hmx3SU4 | scl17a6a/b | 3 | 0.243* |
| NS   | hmx3aSU3 (86.4 ± 0.8) vs. hmx2/hmx3SU4 | scl17a6a/b | 11 | 0.559* |
| NS   | hmx3aSU4 (85.2 ± 1.2) vs. hmx2/hmx3SU4 | scl17a6a/b | 41 | 0.126* |
| NS   | hmx3aSU4 (85.2 ± 1.2) vs. hmx2/hmx3SU4 | scl17a6a/b | 41 | 0.126* |
| 6K   | WT (69.6 ± 0.7) vs. hmx3aSU3 | hmx3a | 11 | 0.613* |
| 6L   | WT (47.6 ± 2.0) vs. hmx3aSU3 | en1b | 3 | 0.356* |
| 6M   | WT (154.4 ± 2.9) vs. hmx3aSU3 | scl3a21 | 13 | 0.004 |
| 6N   | WT (158.0 ± 2.0) vs. hmx2/hmx3SU4 | scl3a21 | 11 | 0.002 |
| NS   | WT (158.0 ± 1.3) vs. hmx3aSU3 | scl3a21 | 10 | 0.012 |
| 6O   | WT (130.4 ± 1.4) vs. hmx3aSU3 | scl6a5 | 0 | 0.907 |
| NS   | WT (111.8 ± 3.9) vs. hmx2/hmx3SU4 | scl6a5 | 7 | 0.234 |
| 6AB  | WT (79.0 ± 2.1) vs. hmx3aSU3 | gad1b | 11 | 0.022 |
| 6AC  | WT (77.5 ± 2.5) vs. hmx2/hmx3SU4 | gad1b* | 10 | 0.014 |
| 6AB and AC | hmx3aSU2 (90.4 ± 2.4) vs. hmx2/hmx3SU4 | gad1b* | 3 | 0.382 |
| 6AD  | WT (151.4 ± 2.7) vs. hmx3aSU3 | gad1b (48hph)* | 17 | 0.001 |
| 6AE  | WT (143.5 ± 6.7) vs. hmx2/hmx3SU4 | gad1b (48hph)* | 27 | 0.02* |
| 6AF  | WT (152.4 ± 4.8) vs. hmx2SU39 | gad1b (48hph)* | 1 | 0.608 |
| 6AD and AE | hmx3aSU3 (175.7 ± 3.5) vs. hmx2/hmx3SU4 | gad1b (48hph)* | 5 | 0.562 |

Statistical comparisons between WT sibling embryos and mutant embryos. First column indicates the figure panel that contains the relevant individual value plots for the comparison. Second column states the genotypes being compared. Single mutant and double deletion embryos have to be obtained from different parents because *hmx2* and *hmx3a* are adjacent to each other on chromosome 17. Therefore, these were always analyzed as separate experiments and single mutants were only compared to double deletion mutants when there was no statistically significant difference between the WT sibling cell counts in the two experiments. Numbers within parentheses indicate mean numbers of cells ± SEM. In all cases, numbers are an average of at least five embryos and cells were counted in all dorsal-ventral spinal cord rows. All of the experiments were conducted on 27 hpf embryos except those indicated with (48 hpf) next to the gene name, which used 48 hpf embryos. 27 hpf embryos fixed on different days varied slightly in stage from prim-stage 9–12. This explains the small differences in numbers of cells labeled with a particular probe in WT embryos in different experiments. Column three lists the gene that the cell counts and statistical comparison refer to. Asterisks indicate experiments performed with dextran sulfate (see Materials and Methods). The fourth column indicates the difference between the two mean values for the embryos being compared. All values are rounded to the nearest whole number. Last column shows the P-value for the comparison, rounded to three decimal places. Statistically significant (P < 0.05) values are indicated in bold. Statistical test used is indicated by superscript symbol: Wilcoxon–Mann–Whitney test (*), type 2 Student’s t-test (**), or type 3 Student’s t-test (**). For a discussion of why particular tests were used, see Materials and Methods: NS, not shown in a figure; †, increase; ‖, decrease.

*hmx3a*, and *hmx2/hmx3a* CRISPR mutagenesis and genotyping. Microarray data have been previously deposited in the NCBI Gene Expression Omnibus under accession number GSE145916.

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far caudally (Figure 1, B and C). Consistent with previous reports, both of these genes are also expressed in the lateral line and developing ear (Figure 1; Adamska et al. 2000; Feng and Xu 2010; Hartwell et al. 2019) as well as distinct regions of the brain (data not shown).

As hmx3a is expressed in the spinal cord, and teleost duplicate genes often have similar expression patterns, we wanted to further test whether there was any spinal cord expression of hmx3b. To investigate this possibility, we performed in situ hybridization on mindbomb1 (mb1<sup>1628</sup>) mutants at 24 hpf. mb1 encodes an E3-ubiquitin protein ligase required for efficient Notch signaling. Consequently, Notch signaling is lost in mb1<sup>1628</sup> mutants and this causes most spinal progenitor cells to precociously differentiate into early-forming classes of spinal neurons at the expense of later-forming classes of neurons and glia (Jiang et al. 1996; Schier et al. 1996; Itoh et al. 2003; Park and Appel 2003; Batista et al. 2008). As a result, weak expression in spinal neurons is often expanded and stronger, and hence easier to observe, in 24 hpf mb1<sup>1628</sup> mutants (Batista et al. 2008; England et al. 2017). However, even in mb1<sup>1628</sup> mutants we detected no expression of hmx3b (Figure 1I). We also analyzed the expression of the other hmx genes in 24 hpf mb1<sup>1628</sup> mutants. The expression patterns of both hmx2 and hmx3a are expanded in the spinal cord of these mutants (Figure 1, G and H), but are unaltered in the ear and lateral

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**Figure 1** Expression of hmx genes in WT zebrafish embryos. Lateral views of hmx expression in spinal cord (A–AD), hindbrain (S’, X’, AC’), eye and ear (A’, E’, F’, J’, K’, O’, P’, T’, U’, Y’, Z’, AD’), and lateral line primordium and neuromasts (B, C, L, M, Q, R, V, W) at 19 hpf (A–E, A’, E’), 24 hpf (F–J, F’, J’), 27 hpf (K–O, K’, O’, P, P’, T, T’, U, U’, Y, Y’, Z, Z’, AD, AD’), 36 hpf (F–J, F’, J’), 42 hpf (U–Y, U’, X’, Y’), and 48 hpf (Z–AD, Z’, AC’, AD’). Rostral, left; Dorsal, up. (B, C, G, H, L, M, Q, R, V, W, AA, AB) hmx2 and hmx3a are expressed in spinal cord, lateral line primordium (white asterisks), neuromasts, and anterior ear (data not shown) at all stages examined, although hmx2 spinal cord expression initially appears weaker than hmx3a and does not extend as far caudally. While there is expression of both hmx2 and hmx3a in the lateral line primordium at 24 hpf (data not shown), the lateral line primordium has not yet migrated into the field of view shown in G and H. For consistency, the specific region of spinal cord shown (adjacent to somites 6–10) is identical in panels F–AD. At 19 hpf, expression is found only in the very anterior spinal cord and so a more rostral region of spinal cord is shown in A–E. (A, A’, E’, F’, F’, J’, K’, O, O’, P, P’, T, T’, U, U’, Y, Y’, Z, Z’, AD, AD’). hmx1 and hmx4 are not expressed in WT spinal cord at any of these stages. The only expression we observed was in the hindbrain between 36 and 48 hpf (black arrows). (G and H) The expression pattern of hmx2 and hmx3a is expanded in the spinal cord of mb1<sup>1628</sup> mutants but is unaltered in the ear and lateral line primordium (data not shown). (F, F’, J’, J’). Neither hmx1 (F) nor hmx4 (J) are expressed in the spinal cord of mb1<sup>1628</sup> mutants, although the expression of both genes persists in the eye (black arrowheads), posterior-ventral ear and adjacent ganglion of the posterior lateral line (white arrowheads). (D, I, N, S, S’, X, X’, AC, AC’) hmx2b is not expressed in WT spinal cord at any of these stages. The background (diffuse, nonspecific staining) in these pictures is higher because we exposed the embryos to prolonged staining to ensure that there was no weak spinal cord expression. Especially in the brain, this can lead to background staining as the large ventricles of the hindbrain trap anti-sense riboprobes. Bar, 50 μm (A–AD), 120 μm (A’, E’, F’, J’, K’, O’, P’, S’, T’, U’, X’, Y’, Z’, AC’, AD’).
The expanded spinal cord expression of hmx2 and hmx3a in mib1ta52b mutants (Figure 2a). Approximately half of these hmx2 and hmx3a (hmx2/3a) coexpressing spinal cells also coexpress slc32a1, which is only expressed by inhibitory (glycinergic and GABAergic) interneurons (Jellali et al. 2002), and approximately half coexpress slc17a6a/b, which are only expressed by excitatory (glutamatergic) interneurons (Serrano-Saiz et al. 2013) (Figure 2b, B and D; see Materials and Methods for a more detailed description of probes used to determine neurotransmitter phenotypes and additional references). In addition, the inhibitory hmx2/3a-expressing cells are generally more ventral than the excitatory double-labeled cells. Our previous expression-profiling of V1 interneurons, suggested that these cells might be the ventral inhibitory neurons that express hmx3a (Figure 2G; for a description of these experiments see Cerda et al. (2009)). Results from our laboratory and others have established that V1 interneurons are the only spinal cord cells that express engrailed1b (en1b) (Higashijima et al. 2004a; Batista and Lewis 2008). Therefore, to confirm that V1 interneurons also express hmx3a, we performed double in situ hybridizations for hmx3a and en1b. These experiments showed that all of the en1b-expressing spinal cells coexpress hmx3a, and that approximately half of the hmx2/3a-expressing spinal cells coexpress en1b (Figure 2C). Taken together, these data clearly identify the inhibitory hmx2/3a-expressing spinal cells as V1 interneurons.

As mentioned above, the glutamatergic hmx2/3a-expressing cells are generally located more dorsal to the inhibitory hmx2/3a-expressing cells. Therefore, these excitatory cells could be V0v, dI5, dI3, dI2, or dI1 interneurons (Figure 2;
Cheng et al. 2005; Grossmann et al. 2010; Satou et al. 2012; Talpalar et al. 2013). The zebrafish embryonic spinal cord is relatively small. For example, at 27 hpf, the dorsal-ventral axis is only about 10 cells high. As a result, the different neuronal populations are often intermingled, rather than clearly separated as they are in amniotes (e.g., Batista et al. 2008; England et al. 2011). In addition, studies in amniotes suggest that many dorsal neurons migrate dorsally or ventrally soon after they are born (e.g., Gross et al. 2002; Müller et al. 2002). Taken together, this means that it is hard to accurately identify dorsal spinal cord cell types by position alone. Therefore, to identify the excitatory hmx2/3a-expressing neurons, we performed double-labeling experiments with markers of different dorsal excitatory cell types. We found that all hmx2/3a-expressing spinal cells coexpress lhx1a and lhx5 (Figure 2, E and G). Lhx1a and Lhx5 are predominantly expressed by inhibitory spinal cord interneurons, but they are also expressed by dI2 interneurons, which are excitatory (Gowan et al. 2008; England et al. 2011). Interestingly, we also did not see abnormal lateral line progression or ear phenotypes in SKD embryos.

To confirm the specificity of these morpholino knockdown results, we first tested whether we saw a similar spinal cord phenotype if we injected splice-blocking morpholinos against hmx2 and hmx3a (see Materials and Methods). In these experiments we obtained a partial reduction in the correct splicing of these genes (Figure S1, A–D) and a statistically significant reduction in the number of glutamatergic spinal cord cells (Figure S1E and see details in the figure legend). The reduction in the number of glutamatergic cells was less than for the translation-blocking DKD experiments, (14 vs. 34 cells; see Figure 3M, Figure S1E, and Table 1), consistent with the fact that we only obtained a partial knockdown of each gene using the splice-blocking morpholinos. We then tested whether co-injecting a morpholino-resistant hmx2 or hmx3a mRNA with the translation-blocking morpholinos could rescue the reduction in the number of spinal glutamatergic cells that occurs in SKD and DKD embryos (see Materials and Methods for the design of the mRNAs). We found that both hmx3a and hmx2 morpholino-resistant mRNA could completely rescue the translation-blocking morpholino phenotype in hmx2 SKD embryos (Figure 3, T–V and Table 1) and hmx3a could completely rescue and hmx2 could partially rescue the translation-blocking morpholino phenotype in hmx3a SKD embryos (Figure 3, W–Y and Table 1). In addition, either hmx2 or hmx3a morpholino-resistant mRNA was able to partially rescue the number of glutamatergic spinal neurons in DKD embryos (Figure 3, Q–S and Table 1). Injections of higher amounts of mRNA or of both mRNAs at the same time led to embryo death, probably because of the toxic effects of injecting considerable amounts of both mRNA and morpholinos into the embryos during early development.

Mutational analyses suggest that hmx2 is not, by itself, required, and that Hmx3a protein may not require its DNA-binding homeodomain for viability, correct migration of lateral line primordium, or correct development of ear otoliths or a subset of spinal cord interneurons

To further and more robustly test the hypothesis that hmx2 and hmx3a are required for the correct specification of a medio-ventral region of each ear (Figure 3D). When we analyzed spinal cord phenotypes, we detected no change in the number of hmx3a- or en1b-expressing cells in DKD embryos, suggesting that dI2 and V1 interneurons still form in normal numbers (Figure 3, E–J and Table 1). However, when we examined markers of neurotransmitter phenotypes, we observed a reduction in the number of spinal excitatory (glutamatergic, slc17a6-expressing) cells and a corresponding increase in inhibitory (slc32a1-expressing) cells (Figure 3, K–P and Table 1). As hmx2 and hmx3a are only expressed by dI2 neurons (which are glutamatergic) and V1 neurons (which are inhibitory) in the spinal cord, this suggested that at least some dI2 interneurons had switched their neurotransmitter phenotype from glutamatergic to inhibitory. Consistent with the idea that the two genes act redundantly, both of these spinal cord phenotypes were less severe in SKD embryos (Figure 3, M and P and Table 1).

Knockdown experiments suggest that hmx2 and hmx3a may be redundantly required for correct specification of a subset of spinal interneuron glutamatergic phenotypes

As an initial step to try and identify the function(s) of hmx2 and hmx3a in spinal cord development we performed morpholino knockdown experiments. As previous analyses suggested that these genes have redundant roles in ear and lateral line development (Feng and Xu 2010), we designed and injected translation-blocking morpholinos for both of these genes (see Materials and Methods). In embryos co-injected with the two translation-blocking morpholinos (hmx2;hmx3a DKD animals), we observed stalled lateral line progression and fused otoliths in the ears (Figure 3, A–D). Normally there is an anterior (utricular) and a posterior (saccular) otolith in each ear, but in DKD embryos there was just one fused otolith in a
Figure 3  *hmx2;hmx3a* double knockdown (DKD) embryos have fewer excitatory (glutamatergic) and more inhibitory spinal cord interneurons. (A–D, E, F, H, I, K, L, N, O, Q, R, T, U, W, X) Lateral views of (A, B, E, F, H, I, K, L, N, O, Q, R, T, U, W, X) spinal cord at 27 hpf and (C and D) otic vesicles at 3 d. Rostral, left; Dorsal, up. (G, J, M, P, S, V, Y) Mean number of cells expressing *hmx3a* (G), *en1b* (J), *slc17a6a/b* (M, S, V, Y), and *slc32a1* (P) in a precisely defined spinal cord region adjacent to somites 6–10 at 27 hpf. All counts are an average of at least five embryos. Data are depicted as individual value plots and the n-values for each genotype are also shown. For each plot, the wider red horizontal bar depicts the mean number of cells and the red vertical bar depicts the SEM (SEM values are listed in Table 1). Statistically significant (P < 0.001) comparisons are indicated with brackets and three asterisks. All data were first analyzed for normality using the Shapiro–Wilk test. Data set in G is nonnormally distributed and was, therefore, analyzed with the Wilcoxon–Mann–Whitney test. Data sets in J, M, P, S, V, and Y are normally distributed and so, for the pairwise comparison shown in J, the ...)
subset of spinal cord interneuron neurotransmitter phenotypes, we created CRISPR mutants in each of these genes, targeting a region upstream of the homeobox (see Materials and Methods; Figure 4). We also obtained a hmx3a<sup>sa23054</sup> allele from the Sanger zebrafish mutation project (Kettleborough et al. 2013) that introduces a stop codon upstream of the homeobox.

Our analyses of homozygous mutant embryos demonstrate that hmx3a<sup>SU3</sup> and hmx3a<sup>SU43</sup> mutants have fused otoliths, stalled lateral line progression, and are homozygous lethal (Figure 4, Figure 5, O and U, Table 3 and data not shown; also see Hartwell et al. (2019) for a detailed description of the hmx3a<sup>SU3</sup> ear phenotype). When we examined the spinal cords of these mutants, we observed a statistically significant reduction in the number of glutamatergic cells, but in both cases the reduction was smaller than we had previously observed for morpholino-injected DKD embryos (Figure 5, D and I and Table 2). There was also an increase in the number of inhibitory spinal cord interneurons; although, again, the increase was less than in the DKD morpholino-injected embryos (Figure 6, C, H and M and Table 2). However, similar to the DKD embryos, there was no change in the number of spinal hmx3a- or en1b-expressing cells in hmx3a<sup>SU3</sup> mutants, suggesting that dI2 and V1 interneurons are forming in normal numbers and not dying or changing into different classes of interneurons (Figure 6, A, B, F, G, K and L, and Table 2).

In contrast, embryos homozygous for hmx3a<sup>SU42</sup> do not have fused otoliths or stalled lateral line progression (Figure 4, Figure 5M, and Table 3) and, unlike hmx3a<sup>SU3</sup> mutants, they have normal expression of hmx3a in the anterior otic epithelium and adjacent anterior neuroblasts (Figure 5S and cf. Figure 5, R and U). hmx3a<sup>SU42</sup> mutants also have normal numbers of spinal cord glutamatergic neurons (Figure 5, B and G and Table 2) and are homozygous viable (Figure 4 and Table 3). Interestingly, embryos homozygous for hmx3a<sup>sa23054</sup> have variable, incompletely penetrant otolith fusion phenotypes that range from no fusion, through incomplete fusion (Figure 5T), to complete fusion, despite the fact that any protein encoded by this allele should retain more WT sequence than that encoded by hmx3a<sup>SU42</sup> (Figure 4). However, hmx3a<sup>sa23054</sup> mutants have normal lateral line progression, no reduction in the number of spinal cord glutamatergic neurons and they are also viable (Figure 4, Figure 5, C, H and N, Table 2, and Table 3).

Surprisingly, we also found that all four of the different hmx2 alleles we created in these experiments (hmx2<sup>SU35</sup>, hmx2<sup>SU36</sup>, hmx2<sup>SU37</sup>, hmx2<sup>SU38</sup>) are homozygous viable and have no obvious defects in otolith development. We also examined lateral line progression and the number of spinal cord glutamatergic neurons in hmx2<sup>SU37</sup> and hmx2<sup>SU38</sup> homozygous mutants and did not detect any change compared to WT embryos (Figure 4, Table 2, and Table 3, and data not shown).

To test whether our hmx2 mutants had no obvious phenotypes because they had retained some Hmx2 function, we created a large deletion allele, hmx2<sup>SU39</sup>, that deletes most of the hmx2 genomic sequence. Only 84 nucleotides of 5′ and 60 nucleotides of the most 3′ coding sequence remain (Figure 4). However, hmx2<sup>SU39</sup> homozygous mutants also lack fused otoliths and are homozygous viable. They also have normal expression of hmx3a and pax5 in the anterior otic epithelium, normal expression of hmx3a in the adjacent anterior neuroblasts, the normal complement of three distinct cristae and two distinct maculae in the ear, and normal lateral line progression (Figure 4, Figure 5, Q, W, AA, AE and AI, Table 2, and Table 3). In addition, there is no change in the number of glutamatergic cells in the spinal cords of these mutants, F-test for equal variances was performed. This data set has equal variances and so a type 2 (for equal variances) Student test was performed. To accurately compare the four different data sets each shown in panel M, P, S, V, and Y, a one-way ANOVA test was performed. All data sets for ANOVA analysis have both normal distributions and homogeneous (homoscedastic, Bartlett’s test P > 0.05) variances and so standard ANOVA analysis was performed. All ANOVA analyses shown are significant [M: ANOVA F(3,76) = 231.5, P = 0.0001; P: ANOVA F(3,19) = 80.64, P = 0.0001; S: ANOVA F(3,76) = 196.3, P < 0.0001; V: ANOVA F(3,56) = 34.97, P < 0.0001; and Y: ANOVA F(3,56) = 61.14, P < 0.0001], and so to determine which specific experimental group or groups differed, Tukey’s honestly significant difference post hoc test for multiple comparisons was performed. Mean numbers of cells and P-values are provided in Table 1. In some cases, cell count data were pooled from different experiments (uninjected control data in M, S, V, and Y from 12 pooled experiments, hmx2/hmx3a DKD data in M and S from 4 pooled experiments, hmx2 SKD data in M and V from 2 pooled experiments, and hmx3a SKD data in M and Y from 2 pooled experiments). As in situ hybridization staining can vary slightly between experiments, we only pooled data from different experiments, or compared different morpholino injection experiments if pairwise comparisons of the counts from corresponding uninjected WT control embryos were not statistically significantly different from each other. (A and B) By 27 hpf, in an uninjected WT control embryo krt15 mRNA expression shows that the lateral line primordium (LLP) has migrated to its expected position over somite 10 (S10 + black arrow) (A). In contrast, at 27 hpf in hmx2/hmx3a DKD embryos, the LLP is stalled beside somites 1-4 (S1, S4, black arrows, B). This is identical to the stalled LLP phenotype observed in hmx3a<sup>SU3</sup> and hmx2/hmx3a<sup>SU44</sup> mutants (see Figure 5, O and P). Dotted line indicates dorsal spinal cord boundary in A and dorsal posterior hindbrain and anterior spinal cord boundary in B. (C and D) Also like hmx3a<sup>SU41</sup> and hmx2/hmx3a<sup>SU44</sup> mutants (see Figure 5, U and V), hmx2/hmx3a DKD embryos have fused otoliths at 3 d (D), but uninjected controls have not (C). (E-J) There is no change in the number of hmx3a- or en1b-expressing spinal cells in hmx2/hmx3a DKD compared to uninjected control embryos, suggesting that V1 and dI2 interneurons do not die or transdifferentiate into different cell types. (K-M) The number of slc17a6a-expressing (excitatory) spinal cells is reduced in both double and single knockdown (SKD) embryos, with the reduction being more severe in DKD embryos. (N-P) Concomitantly, there is a statistically significant increase in the number of slc32a1-expressing (inhibitory) cells in both SKD and DKD embryos, with the increase being more profound in DKD embryos. (Q-S) Injection of either morpholino-resistant hmx2 (Q) or hmx3a mRNA (R) partially rescues the number of spinal excitatory cells in DKD embryos. (T-V) Injection of either morpholino-resistant hmx2 (T) or hmx3a mRNA (U) fully rescues the number of spinal excitatory cells in hmx2 SKD embryos. (W-Y) Injection of morpholino-resistant hmx2 mRNA (W) partially rescues the number of spinal excitatory cells in hmx3a SKD embryos, but injection of morpholino-resistant hmx2a mRNA (X) fully rescues the phenotype. Bar, 30 μm (A, B, E, F, H, I, K, L, N, O, Q, R, T, U, W, and X); 80 μm (C and D).
When compared to WT sibling embryos (Figure 5, F and K and Table 2).

While this was surprising, we hypothesized that the lack of obvious phenotypes could be because Hmx3a protein was compensating for loss of Hmx2 protein. To test this hypothesis, we created double mutant embryos. As hmx2 and hmx3a are adjacent on chromosome 17, it is not possible to create double mutants by breeding single mutants. Both mutations have to exist on the same chromosome. Therefore, we created two different deletion alleles, hmx2;hmx3aSU44 and hmx2;hmx3aSU45, that lack the entire hmx3a coding sequence and all but the last 66 nucleotides of hmx2 coding sequence (see Figure 4 and Materials and Methods; the alleles differ only in the amount of remaining sequence upstream of

![Diagram](image-url)
Figure 5 Only some hmx2/3a alleles have mutant phenotypes. (A–F, L–AI, L’, R’) Lateral views. Rostral, left; Dorsal, up. (A–F) Expression of slc17a6a/b in spinal cord at 27 hpf. (G–K) Number of cells expressing slc17a6a/b in a precisely defined spinal cord region adjacent to somites 6–10 at 27 hpf. Data are depicted as individual value plots and the n-values for each genotype are also shown. For each plot, the wider red horizontal bar depicts the mean number of cells and the red vertical bar depicts the SEM (SEM values are listed in Table 2). All counts are an average of at least five embryos. Statistically significant (P < 0.001) comparisons are indicated with brackets and three asterisks. White circles indicate WT data and black circles the appropriate mutant data as indicated in key under panel A. All data were first analyzed for normality using the Shapiro–Wilk test. Data in G is not normally distributed and so a Wilcoxon–Mann–Whitney test was performed. Data sets in H–K are normally distributed and so the F-test for equal variances was performed, followed by a type 2 Student’s t-test (for equal variances). P-values are provided in Table 2. (L–Q, L’) Lateral line primordium phenotypes examined either by hmx3a expression (L’, M, N, Q) or live (L, O, P) at 27 hpf. (R–AI, R’, U’) Ear phenotypes examined either by hmx3a expression at 27 hpf (R, S, U’, W), live at 4 d (R’, T–V), pax5 expression at 24 hpf (X–AA) or phalloidin staining at 4 d (AB–AI). (A–K). There is no change in the number of spinal excitatory neurons in hmx3aSU42 (B and G) and hmx3asa23054 (C and H) and hmx2SU39 (F and K) mutant embryos compared to WT (A). In contrast, there is a statistically significant reduction in hmx3aSU3 (D and I) and hmx2;hmx3aSU44 (E and J) mutant embryos. (L–Q, L’). At 27 hpf, the tip of the lateral line primordium (black dotted line, L) has reached somite 10 (S10) in WT embryos (L and L’). This rate of migration is unchanged in hmx3aSU42, hmx3asa23054, and hmx2SU39 mutant embryos (M, N, Q). (O and P) In contrast, the lateral line primordium fails to migrate in hmx3aSU3 (O) and hmx2;hmx3aSU44 (P) mutant embryos. Instead, it is stalled adjacent to somites one to four (S4, somite 4). (R, S, U’, W) hmx3a expression in the ear (inside white dotted lines) and in presumptive neuroblasts anterior to the ear (white arrowheads) is unchanged in hmx3aSU42 (S) and hmx2SU39 (W) mutants, compared to WT embryos (R), but is severely reduced in both the presumptive neuroblasts anterior to the ear and the anterior ear (black arrowhead) in hmx3aSU3 mutants (U’). (T) hmx3asa23054 mutants show incompletely penetrant, variable otolith fusion phenotypes, ranging from no fusion (like WT ear in R’), through incomplete fusion (T), to complete fusion, like that observed at full penetrance in hmx3aSU3 (U) and hmx2;hmx3aSU44 (V) mutants. (X–AA) The expression of pax5 in the anterior ear (inside black dotted lines) is unchanged in hmx2SU39 (AA) and hmx2;hmx3aSU44 (V) mutants. (Y) and hmx2;hmx3aSU44 (Z) mutants. (AB–AI) The three cristae of the ear (white arrowheads) form normally in WT (AB), hmx3aSU3 (AC), hmx2;hmx3aSU44 (AD), and hmx2SU39 (AE) mutants. In contrast, the spatially distinct anterior (utricular, red arrow) and posterior (saccular, white arrow) maculae are unchanged in hmx2SU39 (AI) mutants, compared to WT embryos (AF), but are fused and are located in a more medio-ventral position (white cross) in hmx3aSU3 (AG) and hmx2;hmx3aSU44 (AH) mutants. However, there are no obvious differences between hmx3aSU3 and hmx2;hmx3aSU44 mutants. Bar, 50 μm (A–F), 30 μm (L–W), 20 μm (X–AA), 60 μm (L’, R’, U’, AB–AI). Panels X, Y, AB, AC, AF, and AG are reproduced from Hartwell et al. (2019) as per the Creative Commons Attribution (CC BY) license at PLoS Genetics.
the hmx3a locus). However, in embryos homozygous for these large deletion alleles, the reduction in the number of excitatory spinal cord interneurons and the increase in the number of inhibitory spinal neurons is equivalent to that in hmx3aSU3 and hmx3aSU44 single mutants (Figure 5, E and J, Figure 6, D, I and N, and Table 2). There are also no obvious differences in the otothion fusion or lateral line progression phenotypes between these single mutants and embryos homozygous for the double deletion alleles (Figure 5, P and V, cf. Figure 5, O and U). To further interrogate whether there might be subtle differences in ear phenotypes between hmx3aSU3 and double deletion mutants, we examined the expression of pax5 in the anterior otic epithelium and the presence and integrity of the cristae and maculae of the ear using phalloidin staining. Both the reduction in pax5 expression in the anterior otic epithelium (cf. Figure 5, Z and Y), the fusion/juxtaposition of the maculae within a more ventro-medial position in the ear (cf. Figure 5, AH and AG) and the size and number of cristae in the ear (Figure 5, AB–AD) are equivalent in hmx2;hmx3aSU44 double deletion and hmx3aSU3 single mutants.
Table 3 Statistical analyses of whether the frequencies of homozygous mutants that survive to adulthood or have abnormal otolith development or lateral line progression phenotypes are Mendelian

| Mutant allele | % Otolith fusion phenotype | P-value for otolith fusion phenotype | % Survival | P-value for survival | % Stalled lateral line progression | P-value for stalled lateral line progression |
|---------------|---------------------------|-------------------------------------|------------|---------------------|-----------------------------------|---------------------------------------------|
| SU35          | 0% (n = 100; z)           | N/A                                 | 18.03% (n = 61) | 0.234               | 0% (n = 28)                       | N/A                                         |
| SU36          | 0% (n = 100; z)           | N/A                                 | 27.27% (n = 66) | 0.566               | 0% (n = 26)                       | N/A                                         |
| SU37          | 0% (n = 112; z)           | N/A                                 | 20.19% (n = 104) | 0.258               | 0% (n = 48)                       | N/A                                         |
| SU38          | 0% (n = 56; z)            | N/A                                 | 33.30% (n = 54) | 0.214               | 0% (n = 104)                      | N/A                                         |
| SU39          | 0% (n = 49; z)            | N/A                                 | 30.26% (n = 76) | 0.289               | 0.69% (n = 145)                  | <0.001                                      |
| sa23054       | 12.35% (n = 332; z)       | <0.001                              | 20.78% (n = 77) | 0.428               | 0% (n = 124)                      | N/A                                         |
| SU42          | 0% (n = 121; z)           | N/A                                 | 20.00% (n = 65) | 0.388               | 0% (n = 104)                      | N/A                                         |
| SU43          | 24.85% (n = 2370; z)      | 0.850                               | None          | N/A                 | 0.935                            | N/A                                         |
| SU44          | 23.33% (n = 463; z)       | 0.391                               | None          | N/A                 | 19.44% (n = 108)                 | 0.879                                       |
| SU45          | 25.41% (n = 1334; z)      | 0.752                               | None          | N/A                 | 0.995                            | N/A                                         |

Column one indicates the mutant allele. These are listed in the same order as in Figure 4. Columns two, four, and six show the frequency of embryos with otolith fusion phenotypes from crosses of heterozygous (z) or homozygous (mz) parents, the frequency of viable adult homozygous mutants, and the frequency of embryos with stalled lateral line progression, respectively. n indicates the total number of animals examined. Columns three, five, and seven list the P-value, rounded up to three decimal places, for a chi-squared test of the hypothesis that the frequency of embryos with fused otoliths, homozygous mutant adults that are viable, or embryos with stalled lateral line progression, respectively, is Mendelian [25% for crosses of heterozygous parents (z) and 100% for crosses of homozygous parents (mz)]. Statistically significant values are indicated in bold. N/A, not applicable (cases where no homozygous mutants survived, had otolith fusions or stalled lateral line phenotypes).

To determine whether the increase in the number of spinal inhibitory interneurons reflects an increase in glycinergic or GABAergic neurons, we examined the expression of genes expressed exclusively by cells with these inhibitory neurotransmitter phenotypes (slc6a5 for glycinergic and gad1b for GABAergic; see Materials and Methods). While we found no statistically significant difference in the number of spinal cord glycinergic cells in hmx3aSU37 or hmx2:hmx3aSU44 deletion mutants, there was a statistically significant increase in the number of GABAergic spinal cells in hmx3aSU42 and hmx2:hmx3aSU44 deletion mutants, whereas the other two alleles would retain 107% WT amino acids, whereas the other two alleles would retain 107% WT amino acids (Figure 4). However, despite the similarity of these mutant alleles, embryos homozygous for hmx3aSU37 and hmx3aSU43 have fused otoliths, stalled lateral line progression, altered spinal interneuron neurotransmitter phenotypes, and do not survive to adulthood, whereas embryos homozygous for hmx3aSU42 are viable and lack all of these phenotypes. In addition, hmx3aSU43 mutants have variable otolith fusion phenotypes despite retaining more WT sequence than hmx3aSU42 alleles, and embryos homozygous for hmx2SU39, which almost completely deletes the hmx2 coding sequence, have no obviously abnormal phenotypes. Given the surprising nature of these results, we decided to further investigate these alleles by creating trans-heterozygous animals. To do this we performed different pairwise crosses between fish heterozygous for hmx2SU39, hmx3aSU42, hmx3aSU43, hmx3aSU44, and hmx2:hmx3aSU44, and analyzed ear and lateral line development in the resulting embryos.

Interestingly, when we crossed fish heterozygous for hmx2:hmx3aSU44 with fish heterozygous for hmx2SU39, the resulting embryos had normal otolith development and lateral line progression (Table 4). Given that approximately a quarter of these embryos should lack almost all of the coding sequence for both alleles of hmx2 (hmx2:hmx3aSU44) lacks all but the last 66 bp and hmx2SU39 lacks all but the first 84 and last 60 bp of hmx2a coding sequence; see Figure 4) as well as all of the coding sequence for one allele of hmx3a, this demonstrates that one WT allele of hmx3a is sufficient for normal otolith development and lateral line progression.

As expected, as both alleles produce homozygous mutant phenotypes, when we crossed fish heterozygous for hmx3aSU37, hmx3aSU42, and hmx3aSU43 mutant alleles all introduce a frameshift within four nucleotides of each other (nucleotides 319, 320, and 323 of the coding sequence, respectively). Assuming that all three of these alleles are translated into truncated proteins, hmx3aSU37 would retain 106 WT amino acids, whereas the other two alleles would retain 107 WT amino acids (Figure 4). However, despite the similarity of these mutant alleles, embryos homozygous for hmx3aSU37 and hmx3aSU43 have fused otoliths, stalled lateral line progression, altered spinal interneuron neurotransmitter phenotypes, and do not survive to adulthood, whereas embryos homozygous for hmx3aSU42 are viable and lack all of these phenotypes. In addition, hmx3aSU43 mutants have variable otolith fusion phenotypes despite retaining more WT sequence than hmx3aSU42 alleles, and embryos homozygous for hmx2SU39, which almost completely deletes the hmx2 coding sequence, have no obviously abnormal phenotypes. Given the surprising nature of these results, we decided to further investigate these alleles by creating trans-heterozygous animals. To do this we performed different pairwise crosses between fish heterozygous for hmx2SU39, hmx3aSU42, hmx3aSU43, hmx3aSU44, and hmx2:hmx3aSU44, and analyzed ear and lateral line development in the resulting embryos.

Trans-heterozygous crosses suggest that hmx3aSU42 is a hypomorphic allele

hmx3aSU37, hmx3aSU42, and hmx3aSU43 mutant alleles all introduce a frameshift within four nucleotides of each other (nucleotides 319, 320, and 323 of the coding sequence, respectively). Assuming that all three of these alleles are translated into truncated proteins, hmx3aSU37 would retain 106 WT amino acids, whereas the other two alleles would retain 107 WT amino acids (Figure 4). However, despite the similarity of these mutant alleles, embryos homozygous for hmx3aSU37 and hmx3aSU43 have fused otoliths, stalled lateral line progression, altered spinal interneuron neurotransmitter phenotypes, and do not survive to adulthood, whereas embryos homozygous for hmx3aSU42 are viable and lack all of these phenotypes. In addition, hmx3aSU43 mutants have variable otolith fusion phenotypes despite retaining more WT sequence than hmx3aSU42 alleles, and embryos homozygous for hmx2SU39, which almost completely deletes the hmx2 coding sequence, have no obviously abnormal phenotypes. Given the surprising nature of these results, we decided to further investigate these alleles by creating trans-heterozygous animals. To do this we performed different pairwise crosses between fish heterozygous for hmx2SU39, hmx3aSU42, hmx3aSU43, hmx3aSU44, and hmx2:hmx3aSU44, and analyzed ear and lateral line development in the resulting embryos.
with fish heterozygous for hmx3aSU42 we observed Mendelian ratios of embryos with fused otoliths and stalled lateral line progression (Table 4). Similarly, we obtained Mendelian ratios of embryos with fused otoliths and stalled lateral line progression when we crossed fish heterozygous for hmx3aSU3 with fish heterozygous for hmx2:hmx3aSU44, or fish heterozygous for hmx3aSU42 with fish heterozygous for hmx2:hmx3aSU44.

More interestingly, when we crossed fish heterozygous for hmx3aSU42 with fish heterozygous for either hmx2:hmx3aSU44 or hmx3aSU42, we also obtained Mendelian ratios of embryos with fully penetrant otolith fusion phenotypes and stalled lateral line progression, as opposed to the variable otolith fusion phenotypes and normal lateral line progression that occurs in hmx3aSU3 hmx3aSU42 homozygous mutants (cf. Table 3 and Table 4). Some of the embryos with stalled lateral line progression had the strong phenotype that we observe in hmx3aSU3 and hmx2:hmx3aSU44 homozygous mutants, and the rest had a slightly weaker phenotype (Table 4, similar to Figure 6AA). This suggests that while two hmx3aSU3 alleles provide sufficient Hmx3a activity for normal lateral line progression and, in some cases, normal otolith development, this is not the case for either the combination of one hmx3aSU3 and one hmx3aSU42 allele or the combination of one hmx3aSU3 allele over a hmx3a deletion.

Even more surprisingly, when we crossed fish heterozygous for hmx3aSU42 with fish heterozygous for either hmx2:hmx3aSU44 or hmx3aSU3 we also obtained embryos with fused otoliths and stalled lateral line progression, although most of the embryos had the slightly weaker lateral line phenotype mentioned above (Figure 6, AA and AG and Table 4). For the combination of hmx3aSU42 and hmx2;hmx3aSU44, these phenotypes occurred in Mendelian ratios. However, for the combination of hmx3aSU42 and hmx3aSU42, while we observed a Mendelian ratio of embryos with stalled lateral line progression, only 17% of embryos had abnormal otolith phenotypes and, in most of these cases, the otoliths were either adjacent but not fused in both ears or there was an abnormal otolith phenotype in only one ear (Table 4). When we genotyped a subset of these embryos, we found that all of the embryos with abnormal otolith phenotypes (n = 18, 6 embryos each with either fused otoliths in both ears, adjacent otoliths in both ears, or a fused or adjacent otolith phenotype in only one ear) were hmx3aSU3+/+;hmx3aSU42/+ trans-heterozygotes. Interestingly, when we genotyped embryos with WT otolith phenotypes (two normal otoliths per ear, n = 173), 5.20% were actually hmx3aSU3+/+;hmx3aSU42/+ trans-heterozygotes, 29.48% were only heterozygous for hmx3aSU3, 31.79% were only heterozygous for hmx3aSU42, and 33.53% were homozygous WT. Taken together, these data suggest that even though hmx3aSU42 homozygous mutants have no obvious abnormal phenotypes, hmx3aSU42 is a hypomorphic allele: while two alleles of hmx3aSU42 provide sufficient Hmx3a activity for normal otolith development and lateral line progression, one hmx3aSU42 allele combined with either one hmx2:hmx3aSU44 or one hmx3aSU3 allele does not.

**Loss of hmx2 function can enhance hypomorphic hmx3a phenotypes**

Our comparisons of hmx3aSU3, hmx3aSU43, hmx2;hmx3aSU44, and hmx2;hmx3aSU45 mutant phenotypes (Figure 5, Figure 6 and Table 2) suggested that hmx2 does not act redundantly with hmx3a in zebrafish otolith development, lateral line

| Allele combination | % Ear phenotypes | % Embryos | P-value | % Severe phenotype | % Moderate phenotype | % Weak phenotype | % Stalled lateral line progression | % Strong phenotype | % Weaker phenotype |
|--------------------|------------------|----------|---------|-------------------|--------------------|-----------------|-----------------------------------|-------------------|------------------|
| SU39 x SU44        | 0.7%             | 268      | <0.001  | 0.0%              | 0.0%               | 0.7%            | 0.0%                              | 38                | <0.001           |
| SU3 x SU43         | 22.6%            | 623      | 0.165   | 22.5%             | 0.0%               | 0.1%            | 32.4%                            | 37                | 0.250            |
| SU3 x SU44         | 26.8%            | 276      | 0.487   | 26.8%             | 0.0%               | 0.3%            | 19.4%                            | 36                | 0.441            |
| SU43 x SU44        | 28.3%            | 279      | 0.214   | 28.0%             | 0.0%               | 0.3%            | 29.8%                            | 114               | 0.192            |
| sa23054 x SU44     | 22.4%            | 303      | 0.289   | 22.4%             | 0.0%               | 0.0%            | 26.3%                            | 38                | 0.703            |
| SU44               | 22.6%            | 139      | 0.696   | 22.5%             | 0.0%               | 0.1%            | 32.4%                            | 37                | 0.250            |
| SU42 x SU44        | 22.1%            | 208      | 0.337   | 19.7%             | 1.4%               | 1.0%            | 18.9%                            | 37                | 0.703            |
| SU3 x SU42         | 17.1%            | 1794     | <0.001  | 4.7%              | 6.0%               | 6.4%            | 23.3%                            | 192               | 0.614            |

Parents heterozygous for different mutant alleles were mated and otolith and lateral line progression phenotypes were assayed in their progeny. Otoliths were assayed at 3 d by visual inspection down a stereomicroscope. Lateral line progression was assayed by in situ hybridization for krt15 at 27 hpf. Column one indicates the allele combination tested. Column two shows the percentage of embryos with otolith phenotypes, column three indicates the total number of embryos analyzed. In contrast to increases of hmx3aSU3 or hmx3aSU43, where mutant embryos have fused otoliths in both ears ("severe" phenotype), in some trans-heterozygous crosses we observed two additional types of ear phenotypes. Phenotypes were classified as "moderate" if otoliths were adjacent but not fused in both ears, and as "weak" if there was an otolith fusion or adjacent otolith phenotype in only one ear. The percentage of embryos with each of these phenotypes is provided in columns five (severe), six (moderate), and seven (weak). Column eight indicates the percentage of embryos with stalled lateral line progression, and column nine shows the total number of embryos analyzed. In contrast to increases of hmx3aSU3 or hmx3aSU43, where mutant embryos lack any migration of the lateral line primordium along the trunk ("strong" phenotype), in some trans-heterozygous crosses, we observed embryos where the lateral line primordium had migrated slightly more caudally ("weaker" phenotype). Column 11 shows the percentage of embryos with the strong phenotype or lateral line phenotypes was Mendelian and the P-values for these tests are provided in columns 4 or 10, respectively. Statistically significant values are indicated in bold. We also performed a binomial distribution test, using the cumulative distribution function, to test whether the number of embryos from the SU39 x SU44 cross that had fused otoliths was statistically significantly different from zero. P = 0.264 for this test.
progression, or specification of correct spinal interneuron neurotransmitter phenotypes, as complete removal of both genes does not result in more severe phenotypes than loss of just \(hmx3a\) function. However, if loss of \(hmx3a\) function already produces maximal mutant phenotypes, we would not detect stronger phenotypes in embryos homozygous for the double deletion alleles. Therefore, a more sensitive way to test if \(hmx2\) functions in these developmental processes would be to remove \(hmx2\) function in embryos homozygous for a “weaker” hypomorphic \(hmx3a\) mutant allele, such as \(hmx3a_{SU42}\). Unfortunately, we cannot mate fish with the \(hmx2_{SU39}\) and \(hmx3a_{SU42}\) mutant alleles to make double mutants, as \(hmx2\) and \(hmx3a\) are adjacent on the same chromosome, so each mutant allele is tightly linked to a WT allele for the other gene. Therefore, we decided to knockdown \(Hmx2\) function in \(hmx3a_{SU42}\) mutants using CRISPR-mediated mutagenesis.

We injected CRISPR reagents to mutate \(hmx2\) into embryos from an incross of fish that were heterozygous for \(hmx3a_{SU42}\). We used the MENTHU tool to identify a sgRNA target site that should predominantly result in the same 5 bp deletion frameshift allele (Figure 4 and Figure 7C), being generated through microhomology-mediated end joining (Ata et al. 2018; Mann et al. 2019). We also used a two-part crRNA + tracrRNA system + Cas9 protein ribonucleoprotein complex for the injections, as this can produce a high efficiency of biallelic mutations and F0 phenotypes (DiNapoli et al. 2019; S. J. England, A. Kowalchuk, W. E. Haws and K. E. Lewis, unpublished data; Hoshijima et al. 2019). When we did this, we found that at \(\sim 3.5\) d, 28.25% \((n = 807)\) of \(hmx2\) CRISPR-injected embryos had an abnormal otolith phenotype (21.31% had fused otoliths in both ears and 6.94% had fused or adjacent otoliths in only one ear; Figure 7B, cf. to uninjected control, Figure 7A). In comparison, only 0.6% \((n = 670)\) of uninjected embryos and 2% \((n = 347)\) of embryos injected with a CRISPR crRNA ribonucleoprotein complex that we have used successfully to make mutations in an unrelated gene, had abnormal otolith phenotypes. These control experiments were performed at the same time as the \(hmx2\) CRISPR injections, using embryos obtained from the same heterozygous \(hmx3a_{SU42}\) parent fish.

We examined 40 of the \(hmx2\) CRISPR-injected embryos at \(\sim 30\) hpf for lateral line progression phenotypes and then let these embryos develop to \(\sim 3.5\) d, so we could correlate lateral line and otolith phenotypes. A total of 25% of the embryos had strong or medium stalled lateral line progression phenotypes and all of these embryos also developed fused otoliths in both ears (Table 5). A few additional embryos both had a weaker lateral line progression defect (migration of the primordium was only delayed by two or three somites compared to stage-matched injected siblings) and two of these also developed fused otoliths in both ears. When we genotyped these embryos for \(hmx3a_{SU42}\), we found that all of the embryos with fused otoliths were homozygous for \(hmx3a_{SU42}\) (Table 6).

We also genotyped 72 additional \(hmx2\) CRISPR-injected embryos, just over half of which had otolith defects. The vast majority of the embryos with otolith phenotypes were homozygous for \(hmx3a_{SU42}\) (Figure 7B and Table 7; one embryo with fused otoliths in both ears and one embryo with an
Table 5 Lateral line and otolith phenotypes of 40 embryos from hmx3aSU42/+ parents injected with hmx2MENTSUH CRISPR reagents

| Lateral line phenotype | Fused otoliths both ears | Normal otoliths both ears | Total |
|------------------------|--------------------------|---------------------------|-------|
| Strong                 | 6                        | 0                         | 6     |
| Medium                 | 4                        | 0                         | 4     |
| Weak                   | 2                        | 5                         | 7     |
| Normal                 | 1                        | 22                        | 23    |
| Total                  | 13                       | 27                        | 40    |

Embryos from an incross of hmx3aSU42/+ parents were injected with hmx2MENTSUH CRISPR reagents at the one-cell stage and assayed for lateral line primordium and otolith phenotypes at 30 hpf and 72 hpf, respectively. Rows 2–5 show stalled lateral line primordium migration phenotypes: normal = primordium in expected position (over somite 15 at 30 hpf); weak = primordium migration stalled by two or three somites; medium = primordium moderately stalled (over somite 6–7 at 30 hpf); strong = primordium not detected. Columns 2 and 3 show otolith phenotypes. Column 4 shows total number of embryos with each lateral line phenotype. Row 6 shows the total number of embryos with each otolith phenotype.

Table 6 hmx3aSU42 genotypes of the 40 embryos included in Table 5

| Lateral line/otolith phenotype | hmx3aSU42 homozygous | hmx3aSU42 heterozygous | hmx3aSU42 WT | Total |
|-------------------------------|----------------------|------------------------|--------------|-------|
| Strong/fused                  | 6                     | 0                      | 0            | 6     |
| Medium/fused                  | 4                     | 0                      | 0            | 4     |
| Weak/fused                    | 2                     | 0                      | 0            | 2     |
| Normal/fused                  | 1                     | 0                      | 0            | 1     |
| Weak/normal                   | 0                     | 4                      | 1            | 5     |
| Normal/normal                 | 0                     | 16                     | 6            | 22    |
| Total                         | 13                    | 20                     | 7            | 40    |

hmx3aSU42 genotypes of the 40 injected embryos screened for lateral line primordium and otolith phenotypes in Table 5. Rows 2–7 show combinations of lateral line primordium phenotype (normal, weak, medium, and strong, as in Table 5) and otolith phenotype (fused in both ears, two normal otoliths in both ears, as in Table 5). Columns 2–4 show hmx3aSU42 genotypes. Column 5 shows the total number of embryos with each combination of lateral line primordium and otolith fusion phenotypes. Row 7 shows the total number of embryos with each hmx3aSU42 genotype.

otolith defect in one ear only were heterozygous). In contrast, all except one of the 35 embryos that did not have obvious defects in otolith development were heterozygous for hmx3aSU42 or WT (Table 7).

Taken together, these results suggest that we obtained a high efficiency of hmx2 mutations in our injected embryos and that CRISPR-mediated knockdown of Hmx2 causes hmx3aSU42 mutants to have defects in otolith development and lateral line progression. To test this, we sequenced the hmx2 allele from 23 of the embryos that we had genotyped for hmx3aSU42 that had different otolith phenotypes (Table 8). We found that all of these embryos had a substantial frequency of hmx2 nonsense alleles. As predicted by the MENTHU algorithm, the mutated alleles all contained a 5 bp deletion, although some also had additional mismatches in the three bases immediately before the deletion and the location of the deletion differed by 1 bp in a few cases. In all cases, we estimate that at least 60% of the amplified hmx2 sequences were mutant (Table 8 and Figure 7, C and D). In one of the WT embryos that lacked a phenotype, ~90% of the amplified hmx2 sequences were mutant, suggesting that, consistent with the lack of abnormal phenotypes in hmx2SU39 mutants, CRISPR mutagenesis of hmx2 is not sufficient for abnormal otolith development (Figure 7, C and D).

hmx2 and hmx3a are not expressed maternally

One possible explanation for why the spinal cord phenotype is less severe in hmx2/3a deletion mutants than in morpholino-injected DKD embryos would be if hmx2 and/or hmx3a are maternally expressed, as in this case the morpholinos might knockdown both maternal and zygotic function whereas the mutants would only remove zygotic function. In addition, maternal expression of hmx2 might explain the lack of any obvious abnormal phenotypes in hmx2 single mutants. To test this, we performed in situ hybridization for hmx2 and hmx3a at the 16-cell stage. However, we did not detect any maternal expression of hmx2, hmx3a or any of the other hmx genes (Figure 8, A–E). We also performed quantitative RT-PCR for hmx2 and hmx3a on whole embryos at different developmental stages. We did not observe expression of either gene at either the 16-cell stage or at 6 hpf, suggesting that neither hmx2 nor hmx3a are maternally expressed (Figure 8F). At 14 hpf, shortly after when both of these genes start to be expressed in the ear and spinal cord, we observed low levels of expression and, for both genes, as expected, this became more abundant at 27 and 48 hpf (Figure 8F). Finally, we also generated embryos from adults that were homozygous mutant for hmx2SU39, hmx3aSU39, and hmx3aSU23054. However, even though half of the embryos in each of these crosses should have been maternal zygotic mutants, we still did not observe any embryos with fused otoliths (Figure 4 and Table 3).

hmx3b, hmx1, and hmx4 expression is not upregulated in hmx2/hmx3aSU42 deletion mutants

Even though hmx3b, hmx1, and hmx4 are not normally expressed in the spinal cord (Figure 1), it was theoretically possible that they are upregulated in response to the absence, or reduced levels, of either Hmx2 and/or Hmx3a protein function, in which case they could partially substitute for the loss of hmx2 and/or hmx3a. To test this, we performed in situ hybridization for these genes in hmx3aSU39 and hmx2;hmx3aSU44 mutants at 27 hpf. In both cases, we did not observe any spinal cord expression of these genes in either genotyped mutants or their sibling embryos, although, as observed previously in WT embryos (Figure 1), hmx1 and hmx4 were expressed in the eye, ear, and anterior lateral line neuromasts in both mutants and WT sibling embryos (Figure 8, G, J, and K and data not shown). As expected, given the deletion of the entire hmx3a coding sequence and all but the last 66 bp of hmx2 coding sequence in hmx2;hmx3aSU44 mutants (Figure 4), we did not detect any hmx2 or hmx3a transcripts in these mutants (Figure 8, H–I).

hmx2SU39 mutants do not lack a spinal cord phenotype because of genetic compensation

Recent reports have demonstrated that genetic compensation (upregulation of other genes that can compensate for loss of
the mutated gene) can result in loss-of-function mutants having a less-severe phenotype than embryos injected with a morpholino against the same gene (Rossi et al. 2015; El-Brolosy and Stainier 2017; Zhu et al. 2017; Sztal et al. 2018; El-Brolosy et al. 2019; Peng 2019). If this is the case, then morpholino knockdown should have less effect on mutant embryos than on WT sibling embryos, because the morpholino will not affect upregulated compensating genes (e.g., Rossi et al. 2015; Sztal et al. 2018). Therefore, to test whether the lack of a spinal cord phenotype in hmx2
SL39 deletion mutants is due to genetic compensation, we injected the translation-blocking hmx2 morpholino into embryos from a cross of fish heterozygous for hmx2
SL39 and performed in situ hybridization for slc17a6a/b to label glutamatergic spinal cord interneurons. We predicted that if there was genetic compensation in hmx2
SL39 mutants, morpholino-injected WT sibling embryos should have a reduced number of spinal cord glutamatergic interneurons, whereas morpholino-injected hmx2
SL39 homozygous mutant embryos should have normal numbers of these cells. However, if there is no genetic compensation, we would expect a similar frequency of morpholino-injected homozygous mutant and WT embryos to have spinal cord phenotypes and those phenotypes to be roughly equivalent in severity. In contrast, if morpholino-injected homozygous mutant embryos have more severe phenotypes than morpholino-injected WT siblings, this might suggest that hmx2
SL39 is a hypomorphic allele. However, this seemed highly unlikely given that the hmx2 gene is almost completely deleted in this allele (Figure 4) and, concordantly, we do not detect any hmx2 transcripts by in situ hybridization (Figure 8N).

We initially examined the injected embryos down a stereomicroscope and divided them into two groups: those that had an obvious reduction in glutamatergic cells and others that either lacked a phenotype or had a more subtle phenotype. When we genotyped these embryos, in both groups we found homozygous mutant and WT embryos at frequencies that were not statistically significantly different from Mendelian ratios (Table 9). In addition, when we compared the average number of glutamatergic cells in morpholino-injected WT and mutant embryos, there was no statistically significant difference between them, regardless of whether we compared all of the morpholino-injected embryos of each genotype or just compared embryos within the same phenotypic group (Table 10). This suggests that the lack of an abnormal spinal cord phenotype in hmx2
SL39 homozygous mutant embryos is not due to genetic compensation, and that the differences that we observed between the two groups of injected embryos instead probably reflect exposure to different levels of morpholino (see Materials and Methods).

hmx3
SU42 mutant alleles do not lack a spinal cord phenotype because of genetic compensation

We also tested whether the spinal cord phenotype of hmx3a
SU42 mutants is less severe than embryos injected with a hmx3a morpholino because of genetic compensation. As above, we injected the translation-blocking hmx3a morpholino into embryos from a cross of fish heterozygous for hmx3a
SU42 and performed in situ hybridization for slc17a6a/b. When we examined the injected embryos down a stereomicroscope, we were able to separate them into one group that had an obvious reduction in spinal glutamatergic cells and another group that either lacked, or had a more subtle, phenotype. However, when we genotyped the embryos in these two groups, we found similar numbers of homozygous mutant and WT embryos in each group and the frequencies of different genotypes were not statistically significantly different from Mendelian ratios (Table 11). In addition, when we compared the average number of glutamatergic cells in morpholino-injected WT and mutant embryos, there was no statistically significant difference between them, regardless of whether we compared all of the morpholino-injected embryos of each genotype or just compared embryos within the same phenotypic group (Table 12). These data suggest that the lack of an abnormal spinal cord phenotype in hmx3a
SU42 homozygous mutant embryos is not due to genetic compensation and that the differences that we observed between the two groups of injected embryos just reflect exposure to different levels of morpholino (see Materials and Methods)
and mutant embryos, there was no statistically significant difference between the two groups of injected embryos within a particular phenotypic group. The observed ratios of genotypes in each phenotypic class was Mendelian. All P values are rounded up to 2 decimal places.

Methods). Consistent with this, we also do not observe any nonsense-mediated decay (NMD) of hmx3a mRNA in hmx3aSU42 mutants (Figure 8O; NMD has been suggested to play a key role in at least some instances of genetic compensation; El-Brolosy et al. 2019).

hmx3aSU3 mutants also do not have genetic compensation

We also tested whether hmx3aSU3 mutants have a less severe spinal cord phenotype than morpholin knockdown embryos because of genetic compensation. As above, we injected the translation-blocking hmx3a morpholino into embryos from a cross of fish heterozygous for hmx3aSU3 and performed in situ hybridization for slc17a6a/b. When we examined these embryos down a stereomicroscope, some of the embryos appeared to have a severe reduction in glutamatergic cells that resembled the morpholin knockdown phenotype, whereas in the other embryos any reduction was more subtle. However, when we genotyped these embryos, we again found similar numbers of homozygous mutant and WT embryos in both groups and the frequencies of the different genotypes in each group were not statistically significantly different from Mendelian ratios (Table 13). In addition, when we compared the average number of glutamatergic cells in morpholinoinjected WT and mutant embryos, there was no statistically significant difference between them, regardless of whether we compared all of the morpholinoinjected embryos, or just compared the injected embryos within a particular phenotypic group (Table 14). For the “weaker” phenotypic group, the difference between WT and mutant embryos approached statistical significance. However, this is probably because some of the WT embryos in this category had almost no reduction in the number of glutamatergic cells, whereas all of the mutants in this category had at least their normal mutant phenotypes. These results suggest that the differences that we observed between the two groups of injected-embryos probably just reflect exposure to different levels of morpholin (see Materials and Methods), and that embryos in the “weaker” phenotypic group did not receive sufficient morpholin to effectively knockdown hmx3a mRNA or cause the more severe morpholin phenotype. Taken together, these data suggest that the spinal cord phenotype in hmx3aSU3 homozygous mutant embryos is not less severe than the morpholin knockdown phenotype because of genetic compensation. Consistent with this, we also do not detect any NMD of hmx3a mRNA in hmx3aSU3 mutants (Figure 8Q).

Discussion

hmx3a is required for correct neurotransmitter phenotypes of a subset of spinal cord interneurons

In this paper, we identify for the first time, a requirement for hmx3a in spinal cord interneuron development. We demonstrate that hmx2 and hmx3a are expressed by V1 and dI2 interneurons, which is consistent with very recent scRNA-seq data from mouse spinal cord (Delilie et al. 2019). Of these cell types, only dI2 interneurons are glutamatergic. Therefore, the most likely explanation for the reduction in the number of glutamatergic spinal cord cells in hmx3a mutants is that some dI2 interneurons are losing their glutamatergic phenotypes. Given that we also detect a corresponding increase in GABAergic spinal cord cells, but the number of V1 cells (indicated by en1b expression) does not change, it is likely that the dI2 interneurons that are losing their glutamatergic phenotypes are becoming GABAergic instead. Unfortunately, the respective in situ hybridization probes are not strong enough to formally confirm with double-labeling experiments that dI2 interneurons switch their neurotransmitter phenotype from glutamatergic to GABAergic. However, unless Hmx3a is acting in a cell-nonautonomous manner, which we think is unlikely as this protein has a nuclear localization sequence and no obvious signal peptide, this is the most likely explanation of our data.

hmx3a is required for progression of the posterior lateral line primordium

Feng and Xu previously reported that the number of posterior lateral line primordium neuromasts was either severely reduced or completely lost at 3 d in hmx2/3a DKD animals (Feng and Xu 2010). Intriguingly, the few neuromasts that sometimes persisted were located very rostrally in the embryo, close to the earliest-forming somites. Our analyses demonstrate that at 27 hpf, when the posterior lateral line primordium has migrated to somite 10 in WT embryos, in hmx3aSU3, hmx3aSU43, hmx2/hmx3aSU44, and hmx2/hmx3aSU45 mutants the primordium is stalled adjacent to somites one to four. This suggests that the previously reported loss of neuromasts at 3 d is probably caused by the posterior lateral line primordium failing to migrate and deposit neuromasts. Feng and Xu (2010) also described reduced cell proliferation (at 15 hpf) and reduced hmx3a expression (at 24 hpf) in the posterior lateral line primordium of

| Embryo class | WT | Het | hmx2SU39 mutants | N value | P value |
|-------------|----|-----|-----------------|---------|---------|
| Un injected control | 33.3% | 57.6% | 9.1% | 33 | 0.11 |
| WT-like | 44.4% | 22.2% | 33.3% | 9 | 0.12 |
| Morphant-like | 32.3% | 41.9% | 25.8% | 31 | 0.68 |

Spinal cord phenotypes were assessed by slc17a6a/b in situ hybridization. hmx2 translation-blocking morpholino-injected embryos from an incross of hmx2SU39+ parents were visually inspected on a stereomicroscope and categorized as resembling either a “WT-like” (row 3) or “morphant-like” (row 4) spinal cord phenotype, compared to uninjected controls (row 2). Embryos were genotyped to identify homozygous WTs (column 2), heterozygotes (column 3) and homozygous mutants (column 4). Column 5 shows the total number of embryos in each phenotypic class. Column 6 shows the P value from Chi-squared tests performed to assess whether the observed ratios of genotypes in each phenotypic class was Mendelian. All P values are rounded up to 2 decimal places.
hmx2/3a DKD animals. While we cannot rule out the possibility that the lateral line primordium fails to migrate because it has not formed correctly, we observe persistent expression of both hmx3a and krt15 in the stalled primordium of our hmx3aSU3, hmx3aSU43, hmx2;hmx3aSU44, and hmx2;hmx3aSU45 mutants (data not shown), suggesting that some other mechanism, possibly chemosensory, might underlie the stalled migration.

**Hmx3a protein may not require its homeodomain for its functions in viability and otolith, lateral line, and spinal cord interneuron development**

Our results also suggest that Hmx3a protein may not require its homeodomain for either its role in viability or its essential functions in otolith development, lateral line progression, and correct specification of a subset of spinal cord neurotransmitter phenotypes. This is surprising because most homeodomain proteins act as transcription factors and use their homeodomain to bind DNA and regulate gene expression. Instead, our data suggest that there may be at least one other, as yet undiscovered, crucial functional domain in the N-terminal region of Hmx3a, that is required for its functions in embryo development and viability, as embryos homozygous for hmx3aSU42 are viable and have no obvious abnormal phenotypes, and embryos homozygous for hmx3aSU3 and hmx3aSU43 are also viable, have normal lateral line progression, and spinal cord interneuron neurotransmitter phenotypes and produce viable progeny. It is highly unlikely that the lack of obvious abnormal phenotypes in these two different mutants is due to an alternative translation start site creating a truncated Hmx3a protein that contains the homeodomain, as the only downstream methionine in hmx3a is more than a third of the way through the homeodomain, and also, in this case we would expect the hmx3aSU3 and hmx3aSU43 alleles to also make this truncated protein. The lack of obviously abnormal phenotypes in hmx3aSU42 and hmx3aSU3 alleles homozygous mutants also cannot be explained by alternative splicing, as these mutations are in the second of two coding exons, and also, when we sequenced cDNA made from homozygous hmx3aSU42 mutants, we obtained the sequence that we expected (see Materials and Methods). It is still theoretically possible that there is translational read-through in these two alleles and not in the other hmx3a mutant alleles that have obvious abnormal phenotypes. While this seems unlikely given how similar these different alleles are, we cannot rule out this possibility as we have not been able to identify an antibody that is specific to Hmx3a and we could not detect any Hmx3a peptides in SWATH analysis (see Discussion below). However, the most parsimonious explanation of our data so far is that Hmx3a does not need its homeodomain for its functions in viability and otolith, lateral line, and spinal cord interneuron development. In this case, while Hmx3a may still bind to other DNA-binding proteins and function in transcriptional complexes, unless the N-terminal of Hmx3a contains a novel DNA-binding domain, Hmx3a is not acting as a classic transcription factor (defined in the strict sense as a protein that binds DNA and regulates transcription) during these developmental processes. Nevertheless, as the homeodomain is highly conserved, suggesting that it is still under evolutionary pressure to be maintained, it is possible that Hmx3a has additional functions that do require this domain, either in adult fish or in aspects of development that we did not assay. However, if this is the case, it is still striking that these functions are not required for such fundamental processes as embryonic development and adult viability.

There are a few other examples of homeodomain proteins that can function in some contexts without their homeodomain. For example, protein interaction and overexpression experiments suggest that Lbx2 does not require its homeodomain to enhance Wnt signaling during gastrulation in zebrafish embryos (Lu et al. 2014). Instead, it sequesters TLE/Groucho, preventing this protein from binding to TCF7L1 and reducing TLE/TCF corepressor activity. In addition, homothorax (hth) does not require its homeobox for its functions during Drosophila head development and proximo-distal patterning of the appendages, although the homeodomain is required for antennal development (Noro et al. 2006). hth has 16 exons and three alternative splice forms. Only one of these isoforms contains the homeobox, but all three contain a protein interaction domain, called the HM domain, that binds to, and can induce the nuclear localization of, Extradenticle (Noro et al. 2006). However, in contrast to hth, zebrafish hmx3a has only two exons and one splice form. While relatively rare, there are also examples of transcription factors from other families that only need to bind DNA for some of their functions. For example, Scl/Tal1 has both DNA-binding dependent and DNA-binding independent functions in hematopoietic and vascular development (Porcher et al. 1999; Ravet et al. 2004).

**Zebrafish hmx2 may not, by itself, be required for viability or correct development of otoliths, lateral line, or spinal cord neurotransmitter phenotypes**

The experiments described in this paper also show that hmx2 single mutants, with progressively larger deletions of the hmx2 coding sequence from hmx2SU37 mutants (with a
Table 11 Genotypes of embryos from hmx3a<sup>SU42</sup>/+ parents, injected with hmx3a morpholino, with different spinal cord phenotypes

| Embryo class       | WT  | Het  | 25.0% | N value | P value |
|--------------------|-----|------|-------|---------|---------|
| Uninjected control | 22.5% | 52.5% |       | 40      | 0.93    |
| WT-like            | 31.25% | 31.25% | 37.5% | 16      | 0.30    |
| Morphant-like      | 20.5% | 56.4% | 23.1% | 39      | 0.61    |

Spinal cord phenotypes were assessed by sk17a6ab in situ hybridization. hmx3a translation-blocking morpholino-injected embryos from an incross of hmx3a<sup>SU42</sup>/+ parents were visually inspected on a stereomicroscope and categorized as resembling either a “WT-like” (row 3) or “morphant-like” (row 4) spinal cord phenotype, compared to uninjected controls (row 2). Embryos were genotyped to identify homozygous WTs (column 2), heterozygotes (column 3) and homozygous mutants (column 4). Column 5 shows the total number of embryos in each phenotypic class. Column 6 shows the P value from Chi-squared tests performed to assess whether the observed ratios of genotypes in each phenotypic class was Mendelian. All P values are rounded up to 2 decimal places.

52 bp deletion), through hmx2<sup>SU38</sup> mutants (with a 427 bp deletion) to hmx2<sup>SU39</sup> mutants (that lack almost all hmx2 coding sequence), do not exhibit NMD (which can trigger genetic compensation in some circumstances; El-Brolosy et al. 2019, Figure 8, L–N), are viable, and have no obvious otolith, lateral line, or spinal cord interneuron neurotransmitter mutant phenotypes. In addition, hmx2;hmx3a<sup>SU44</sup> and hmx2;hmx3a<sup>SU45</sup> deletion mutants do not have more severe phenotypes than hmx3a<sup>SU3</sup> or hmx3a<sup>SU43</sup> single mutants. These results are surprising because zebrafish hmx2 and hmx3a have very similar expression domains during embryonic development (although the spinal cord expression of hmx3a does briefly precede that of hmx2; Figure 1, B and C), these overlapping expression domains are highly conserved in different vertebrates and studies in other animals suggest that hmx2 and hmx3 often act redundantly during development (Wang et al. 2004; Wang and Lufkin 2005; Wotton et al. 2010). Most notably, previous analyses demonstrated that mouse Hmx2 mutants had defects in ear development, though interestingly these were more severe in ~70% of homozygous mutants than in the other 30%, showing that there was some variability in the requirement for Hmx2 (Wang et al. 2001). In addition, mouse Hmx2:Hmx3 double mutants had more severe ear phenotypes than either single mutant, as well as defects in hypothalamus and pituitary development that were not found in either single mutant and most of the double mutants died around the fifth day after birth, whereas the single mutants were viable (Wang et al. 2004). When considered in combination, these data suggest that mouse Hmx2 has important functions in ear and brain development, although some of these are redundant with Hmx3. In contrast, the only mutational analysis where we detected any function for zebrafish hmx2, was when we introduced hmx2 mutations into hypomorphic hmx3a<sup>SU42</sup> mutants. Taken together, our data suggest that while zebrafish Hmx2 protein can function in otolith and lateral line development, it may only affect the development of these structures in embryos with significantly reduced Hmx3a function (less activity than that provided by one functional hmx3a allele, as embryos trans-heterozygous for hmx2;hmx3a<sup>SU44</sup> and hmx2<sup>SU39</sup> develop normally) but more Hmx3a activity than in hmx3a<sup>SU3</sup> or hmx3a<sup>SU43</sup> mutants.

Our experiments also suggest that the lack of any obvious abnormal phenotypes in hmx2<sup>SU39</sup> single mutants is not due to genetic compensation or maternal expression of hmx genes. One possible explanation for why zebrafish Hmx2 might have a diminished role in development compared to mouse Hmx2 or zebrafish Hmx3a could be if zebrafish Hmx2 has evolved to be less conserved with mouse Hmx2 and Hmx3 than zebrafish Hmx3a. However, a comparison of all four proteins only reveals six residues that are shared between mouse Hmx2 and Hmx3 and zebrafish Hmx3a, but not zebrafish Hmx2, and four of these residues are upstream of the hmx3a<sup>SU3</sup> mutation (Figure S2). Our prior research identified Hmx2 and Hmx3 in all of the different vertebrates that we analyzed, including five different teleost species, and our phylogenetic analyses of these proteins did not suggest that Hmx2 has evolved any faster in zebrafish than in other species, or that Hmx2 has evolved faster than Hmx3 (Wotton et al. 2010). Taken together, these observations suggest that there is still considerable evolutionary pressure to maintain zebrafish Hmx2, which in turn suggests that it should have an important role(s) in zebrafish survival and/or reproduction. Therefore, it is surprising that we did not detect more severe consequences from loss of Hmx2. It is possible that Hmx2 has important functions later in development and/or in aspects of development that we did not assay. However, if this is the case, these functions are not required for viability or reproduction, as even hmx2<sup>SU39</sup> homozygous mutants survive to adulthood and produce viable progeny. It is also possible that hmx2 has important function(s) in adult fish, as our assays would not have detected this. It would be interesting to investigate these possibilities in future studies.

**Very similar hmx3a mutant alleles have different homozygous mutant phenotypes**

It is currently unclear why hmx3a<sup>SU42</sup> retains more WT function than hmx3a<sup>SU44</sup> when both should encode proteins with only 107 WT amino acids. As discussed above, we are confident that this is due to alternative splicing or exon skipping. We also do not observe any NMD of hmx3a mRNA for any of our hmx3a single mutant alleles (Figure 8, O–R; the double deletion mutants lack all hmx3a coding sequence, so there is no mRNA to assess, Figure 8I), so the difference in allelic strength is not due to some of the mutant mRNAs being degraded. However, it is possible that different mutant alleles result in different amounts of truncated protein due to differences in translation efficiency or protein stability. Unfortunately, we were not able to test this as there are currently no antibodies that uniquely recognize Hmx3a and we would require an antibody that recognizes the N-terminal region of Hmx3a that should be conserved in our single mutant Hmx3a proteins. In addition, we were unable to detect any Hmx3a peptides in a SWATH analysis (data not shown; Hmx3a has
also not been detected in other SWATH analyses; Blattmann et al. 2019; Lin et al. 2019), presumably because, like many transcription factors, it is expressed in either two few cells and/or at too low a level.

It is also possible that the overall length of the mutant protein is important for retaining function and that the additional abnormal amino acids after the frameshift but before the premature stop codon in hmx3aSU42 help this allele retain more WT function. A longer protein sequence might facilitate a required protein conformation and/or binding with other proteins or molecules. If this is the case, then it could also explain why Hmx3aSU42 protein (which is predicted to contain 107 WT + 42 abnormal amino acids; Figure 4) appears to retain more WT function than Hmx3aSU32054 protein (which should contain only 118 WT amino acids; Figure 4). Currently, there are no known binding partners of Hmx3a. However, if future analyses identify any it would be interesting to test if they can bind to Hmx3aSU42 and Hmx3aSU13.

Another related possibility is that the different stretches of abnormal amino acids after the frameshift in hmx3aSU3, hmx3aSU42, and hmx3aSU13, might introduce sequences that influence protein stability, degradation, or function. Using a variety of online analysis tools, we did not detect any sumoylation, or PEST sequences in any of the predicted mutant protein sequences and the only ubiquitination motifs that we identified are located in the WT sequence present in all four mutant proteins (Rice et al. 2000; Sarachu and Colet 2005; Brameier et al. 2007; Radivojac et al. 2010; Zhao et al. 2014). However, the eukaryotic linear motif prediction tool identified a monopartite variant of a classic, basically charged nuclear localization signal in Hmx3aSU42 protein that is not present in any of the other predicted mutant protein sequences (Via et al. 2009; Gould et al. 2010; Kumar et al. 2020), although this domain was not detected using default parameters with cnLS Mapper or NuPred (Brameier et al. 2007; Kosugi et al. 2009). This is potentially very interesting as WT Hmx3a has a nuclear localization signal located between amino acids 167–177, overlapping the start of the homeodomain at amino acid 171, which is downstream of the mutations in all of these alleles.

In addition, online tools that identify disordered vs. ordered protein structure suggest that both the WT amino acids in Hmx3aSU2054 that are not found in the other predicted Hmx3a mutant proteins and the non-WT amino acids in the predicted protein product of hmx3aSU42 may provide longer stretches of disordered sequence than are present at the end of the predicted protein products of hmx3aSU3 or hmx3aSU43 (Linding et al. 2003; Dosztányi et al. 2005; Ishida and Kinoshita 2007; Dosztányi 2018; Mészáros et al. 2018; Erdős and Dosztányi 2020). These findings raise the intriguing possibility that hmx3aSU42 might retain Hmx3a function because it can still localize to the nucleus and/or that hmx3aSU2054 and hmx3aSU42 might retain some WT activity because of the disordered sequences at the end of their predicted proteins. As disordered protein regions can switch between disordered and ordered states in the presence of a binding partner, it is tempting to speculate that these disordered stretches at the C-termini of Hmx3aSU42 and Hmx3aSU2054 proteins may still be able to bind proteins essential for Hmx3a function that the other alleles cannot (Mészáros et al. 2018). Investigation of these possibilities is outside the scope of the current study but would be interesting to address in future work.

**hmx2 and hmx3a morpholino injections produce more severe spinal interneuron phenotypes than hmx2 and hmx3a mutants**

Our original morpholino data suggested that all dI2 interneurons might be switching their neurotransmitter phenotypes as the increase in the number of spinal inhibitory cells and the reduction in the number of excitatory spinal cells in DKD embryos were both roughly equal to the number of dI2 interneurons (glutamatergic hmx3a-expressing cells). However, even in our hmx2;hmx3a deletion mutants, the number of cells changing their neurotransmitter phenotypes is lower than this. The differences between hmx3a morpholino-injected embryos and mutant embryos can be seen clearly in our experiment to test whether genetic compensation occurs in hmx3aSU3 homozygous mutants. These data directly compare uninjected mutants with morpholino-injected mutants and WT siblings from the same experiment. While there were a range of morpholino-injected phenotypes, overall the hmx3a morpholino-injected WT and hmx3aSU3 mutant embryos had a more severe reduction of glutamatergic cells than uninjected mutants (Table 14). While it is possible that hmx3aSU3 mutants may be slightly hypomorphic, their spinal cord phenotype is the same as hmx2;hmx3aSU44 mutants, in which the hmx3a coding sequence is completely deleted. Therefore, the more severe phenotypes in some of the hmx3aSU3 mutants injected with hmx3a morpholino cannot be explained by the morpholino removing any residual Hmx3a function. This experiment also suggests that the less severe phenotype in uninjected hmx3aSU3 mutants is not caused by genetic compensation. Consistent with this, we have also shown that this less severe mutant phenotype is not due to other hmx genes being upregulated in these

### Table 12 The lack of spinal cord phenotypes in hmx3aSU42 mutants is not due to genetic compensation

| Embryo class       | WT     | hmx3aSU42 mutants | P value |
|--------------------|--------|------------------|---------|
| Uninjected control | 117.56 ± 2.26 | 116.10 ± 1.38  | 0.58*   |
| WT-like            | 116.80 ± 2.89 | 114.67 ± 2.69  | 0.60*   |
| Morphant-like      | 86.38 ± 2.15  | 88.22 ± 5.07   | 0.74*   |
| All injected       | 98.08 ± 4.58  | 98.80 ± 4.67   | 0.91*   |

The spinal cord phenotypes of embryos (as assessed by sk17a6ab expression) in the distinct phenotypic classes shown in Table 11 were analyzed on a compound microscope while blind to genotype. Values in columns 2 and 3 indicate the mean number of labelled cells ± SEM. Column 4 shows the P values from either a type 2 (*, performed when data was normally distributed and variances were equal), or type 3 Student’s t-test (**, performed when data was normally distributed and variances were unequal) for the comparison of homozygous WT embryos to homozygous hmx3aSU42 mutants for a particular classification (values on same row). All P values are rounded up to 2 decimal places. See Materials and Methods for more information on statistical tests.
mutants or maternal expression of *hmx3a* or any other *hmx* genes. These results are very puzzling. There are several reasons to suggest that the more severe spinal cord phenotype in DKD embryos is not due to nonspecific effects of either the *hmx3a* or *hmx2* morpholino. First, we were able to rescue more glutamatergic spinal neurons in our DKD embryos, with co-injection of either *hmx2* or *hmx3a* morpholino-resistant mRNA, than are lost in any of our mutants (Tables 1 and 2). Second, we were able to fully rescue the *hmx3a* SKD phenotype by co-injecting a morpholino-resistant *hmx3a* mRNA, and the *hmx2* SKD phenotype by co-injecting either a morpholino-resistant *hmx2* mRNA or a morpholino-resistant *hmx3a* mRNA. Third, it is unlikely that the more severe phenotype is due to cell death or a delay in embryo development (which are common nonspecific side effects of morpholino injections), as there was no change in the number of *hmx3a* or *en1b* expressing spinal cord cells in DKD embryos and there was an increase in the number of inhibitory spinal cord interneurons equivalent to the reduction in glutamatergic neurons. Finally, it is also unclear, why a nonspecific effect of a morpholino would exacerbate the real loss-of-function phenotype, causing additional spinal cord interneurons to lose their glutamatergic phenotypes and instead become inhibitory. This suggests that if the more severe morpholino injection phenotypes are due to nonspecific effects of the morpholinos, these nonspecific effects produce an identical phenotype to the specific knockdown effect, namely a switch in neurotransmitter phenotype.

Figure 8 Expression of *hmx* genes in mutant zebrafish embryos and before the midblastula transition. (A–E, G–R) Lateral views of expression in whole embryos at 1.5 hpf (16 cells, A–E) or the spinal cord (G–R) at 27 hpf. (A–E) Animal pole, up. (G–R) Rostral, left; Dorsal, up. (L and M, O and P) White asterisk indicates expression in the lateral line primordium. None of the *hmx* genes are maternally expressed at 1.5 hpf, as assessed by in situ hybridization (A–E), and, in the case of *hmx2* and *hmx3a*, quantitative RT-PCR on whole embryos (F). No maternal expression of *hmx2* and *hmx3a* was detected and zygotic expression was not observed via quantitative RT-PCR until 14 hpf (F). *hmx1* (G), *hmx3b* (J), and *hmx4* (K) are not expressed in the spinal cord of *hmx2;hmx3aSU44* deletion mutants. However, *hmx1* and *hmx4* were still expressed in the head, as shown in Figure 1 (data not shown), confirming that the in situ hybridization experiment had worked. We never detect expression of *hmx3b* in WT embryos at 27 hpf (see Figure 1). As expected, given the deletion of the entire *hmx3a* coding sequence and all but the last 66 bp of *hmx2* coding sequence in *hmx2;hmx3aSU44* mutants (Figure 4), we did not detect any *hmx2* (H) or *hmx3a* (I) transcripts in these mutants. L and M) *hmx2* mRNA does not exhibit nonsense-mediated decay (NMD) in *hmx2SU37* or *hmx2SU38* mutants. (N) In *hmx2SU39* mutants, deletion of all but the first 84 and the last 60 bases of *hmx2* coding sequence (Figure 4) generates a severely truncated *hmx2* transcript that cannot be detected by our *hmx2* ISH probe. Generation of a short ISH probe targeted to the predicted truncated transcript product of *hmx2SU39* mutants also failed to detect *hmx2* expression in these mutants (data not shown). (O–R) *hmx3a* mRNA does not exhibit NMD in *hmx3aSU42* (O), *hmx3aSU43* (Q), or *hmx3aSU43* (R) mutant embryos. Bar, 280 μm (A–E), 50 μm (G–R).
severe phenotypes that we see in morpholino knockdown of the gene usually caused a partially penetrant phenotype for some transcription factors (Topalidou et al. 2011; Zheng and Chalifie 2016). In these cases, mutating the gene usually caused a partially penetrant phenotype in ideal conditions but a more severe phenotype in stressed conditions. In this case, the injection of morpholinos could be such a stressed condition, and this could account for the more severe phenotypes that we see in morpholino knockdown experiments compared to mutational analyses. Future analyses could investigate this possibility by testing if other stressed conditions increase the severity of hmx3aSU3 or hmx2SU39 single mutant or hmx2;hmx3a deletion mutant phenotypes. Even if this is not the case, our results suggest that something other than just nonspecific effects from the morpholinos may be occurring. Therefore, we felt that it was crucial to report this morpholino injection data, as an intriguing and hopefully thought-provoking contribution to the continuing discussion about the pros and cons of using morpholinos to investigate gene function.

**hmx3b has very limited expression during early zebrafish embryogenesis**

The data in this paper also provide the first characterization of zebrafish hmx3b expression. Surprisingly, given the expression of all of the other four hmx genes during zebrafish embryonic development, the only expression of hmx3b that we have been able to detect, is very weak expression in the hindbrain from 36 to 48 hpf (Figure 1, S’ , X’, and AC’). When we performed our earlier analyses of vertebrate NK genes, we did not find hmx3b in either the Zv7 or Zv8 versions of the zebrafish genome (Wotton et al. 2010). It did not appear until Zv9. Interestingly, despite Hmx2 and Hmx3 being closely linked in all vertebrates examined so far, only one hmx2 gene has been found in the zebrafish genome and zebrafish hmx3b is not located within any of the previously described duplicated NK homeobox clusters, including the teleost duplications: it is located on chromosome 12, separate from any other nk genes (Wotton et al. 2010). However, hmx3b is flanked by two genes, bub3 and acadsb, that flank Hmx2 and Hmx3 in the human, mouse, chicken, and xenopus genomes, suggesting that part of the NK cluster previously identified on chromosome 1 may have translocated to chromosome 12. This is maybe not surprising as zebrafish hmx2 and hmx3a have also translocated from the rest of their NK cluster on chromosome 13 to chromosome 17, and zebrafish hmx1 and hmx4 have translocated from the rest of their cluster on chromosome 14 to chromosome 1 (Wotton et al. 2010). It is possible that this translocation and the loss of surrounding sequences may explain the different

| Embryo class    | WT       | hmx3aSU3 | N value | P value |
|----------------|----------|----------|---------|---------|
| Uninjected control | 20.4% | 44 | 0.76 |
| Weaker phenotype | 19.5% | 41 | 0.74 |
| Morphant-like    | 21.7% | 83 | 0.54 |

Spinal cord phenotypes were assessed by sk17a6ab in situ hybridization. hmx3a translation-blocking morpholino-injected embryos from an incross of hmx3aSU39 parents were visually inspected on a stereomicroscope and categorized as resembling either a “weaker” (row 3) or more severe, “morphant-like” (row 4) spinal cord phenotype, compared to uninjected controls (row 2). Embryos were genotyped to identify homozygous WTs (column 2), heterozygotes (column 3) and homozygous mutants (column 4). Column 5 shows the total number of embryos in each phenotypic class. Column 6 shows the P value from Chi-squared tests performed to assess whether the observed ratios of genotypes in each phenotypic class was Mendelian. All P values are rounded up to 2 decimal places.

Table 13 Genotypes of embryos from hmx3aSU39 parents, injected with hmx3a morpholino, with different spinal cord phenotypes

| Embryo class    | WT       | hmx3aSU3 | P value |
|----------------|----------|----------|---------|
| Uninjected control | 106 ± 2.44 | 94.5 ± 2.62 | 0.01* |
| Weaker phenotype | 102.75 ± 2.27 | 96.8 ± 1.77 | 0.05* |
| Morphant-like    | 70.22 ± 3.76 | 74.0 ± 2.75 | 0.72* |
| All injected     | 80.23 ± 4.01 | 81.86 ± 2.78 | 0.74* |

The spinal cord phenotypes of embryos (as assessed by sk17a6ab expression) in the distinct phenotypic classes shown in Table 13 were analyzed on a compound microscope while blind to genotype. Values in columns 2 and 3 indicate the mean number of labelled cells ± SEM. Column 4 shows the P values from either a Wilcoxon-Mann-Whitney test (*, performed when data was not normally distributed) or from a type 2 Student’s t-test (**, performed when data was normally distributed and variances were equal) for the comparison of homozygous WT embryos to homozygous hmx3aSU39 mutants for a particular classification (values on same row). All P values are rounded up to 2 decimal places. See Materials and Methods for more information on statistical tests. Statistically significant values are indicated in bold.

Table 14 The incomplete penetrance of the spinal cord phenotype in hmx3aSU39 mutants is not due to genetic compensation

- **It is also puzzling why hmx2 morpholino-injected SKD embryos have reduced numbers of spinal cord glutamatergic cells and an increase in the number of inhibitory spinal interneurons, while hmx2SU39 mutants do not, and our experiments suggest that this is also not due to genetic compensation. In addition, co-injection of a morpholino-resistant hmx2 mRNA rescues DKD embryos as well as hmx3a co-injection, even though WT hmx2 is not sufficient for normal development in hmx3aSU39 or hmx3aSU3 mutants. The latter result could be explained if the mRNA injection provides higher levels of Hmx2 function than is normally found endogenously. However, the first result is harder to explain. As discussed above, our data suggest that it is unlikely that the hmx2 morpholino has nonspecific effects on neurotransmitter phenotypes in the spinal cord. These results are also not due to cross-reaction of the hmx2 translation-blocking morpholino with hmx3a. The hmx3a and hmx2 translation-blocking morpholino sequences are completely different from each other and there is no homology between the hmx2 translation-blocking morpholino and hmx3a upstream or coding sequence. When we use BLAST on the hmx2 translation-blocking morpholino sequence against the zebrafish genome, the only homology is with hmx2 (25/25 residues) and with intronic sequence for a gene st:dkey-73p2.3 on chromosome 3 (18/25 residues), which is predicted to encode a protein with GTP-binding activity. Similarly, when we use BLAST on the hmx3a translation-blocking morpholino sequence against the zebrafish genome, the only homology is with hmx3a (25/25 residues).

One intriguing alternative possibility that could explain the apparent specificity of the additional phenotype in the morpholino-injected embryos could be that Hmx3a and Hmx2 are acting as fate guarantors, to make the normal neurotransmitter phenotype more robust, as has previously been described for some transcription factors (Topalidou et al. 2011; Zheng et al. 2015; Zheng and Chalifie 2016). In these cases, mutating the gene usually caused a partially penetrant phenotype in ideal conditions but a more severe phenotype in stressed conditions. In this case, the injection of morpholinos could be such a stressed condition, and this could account for the more severe phenotypes that we see in morpholino knockdown
expression pattern of hmx3b compared to hmx3a. For example, in previous analyses we identified three highly conserved non-coding regions in the vicinity of hmx2 and hmx3a (two upstream of hmx3a and one in between hmx3a and hmx2) that are conserved in mammals, frog, and teleosts; Wotton et al. 2010, but none of these regions are present near hmx3b (data not shown).

In conclusion, in this paper we provide the first description of zebrafish hmx3b expression. Our results also identify the spinal cord cells that express hmx2 and hmx3a (dI2 and V1 interneurons) and uncover novel functions for hmx3a in correct specification of a subset of spinal cord neurotransmitter phenotypes and in lateral line progression. Our data suggest that while hmx3a is required for viability, correct otolith development, lateral line progression, and specification of a subset of spinal neurotransmitter phenotypes, hmx2 is not, by itself, required for any of these developmental processes, although it can act partially redundantly with hmx3a in situations where hmx3a function is significantly reduced, but not completely eliminated. Finally, our results also suggest that Hmx3a may not require its homeodomain for its roles in viability or embryonic development. Taken together, these findings significantly enhance our understanding of spinal cord, ear, and lateral line development, and suggest that, intriguingly, more homeodomain proteins may not require their homeodomain for many of their essential functions.

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