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Emx3 Is Required for the Differentiation of Dorsal Telencephalic Neurons

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Abstract

emx3 is first expressed in prospective telencephalic cells at the anterior border of the zebrafish neural plate. Knockdown of Emx3 function by morpholino reduces the expression of markers specific to dorsal telencephalon, and impairs axon tract formation. Rescue of both early and late markers requires low-level expression of *emx3* at the one- or two-somite stage. Higher *emx3* expression levels cause dorsal telencephalic markers to expand ventrally, which points to a possible role of *emx3* in specifying dorsal telencephalon and a potential new function for Wnt/beta-catenin pathway activation. In contrast to mice, where Emx2 plays a major role in dorsal telencephalic development, knockdown of zebrafish Emx2 apparently does not affect telencephalic development. Similarly, Emx1 knockdown has little effect. Previously, *emx3* was thought to be fish-specific. However, we found all three *emx* orthologs in *Xenopus tropicalis* and opossum (*Monodelphis domestica*) genomes, indicating that *emx3* was present in an ancestral tetrapod genome.

Keywords

Danio rerio; cell specification; empty spiracles; forebrain; pallium; homeobox; patterning; prosencephalon; transcription factor

INTRODUCTION

Despite recent advances in our understanding of the role of signals from the anterior neural plate in specifying the telencephalon, relatively little is known about how these signals are interpreted by prospective telencephalic cells. Some factors expressed at the anterior edge of the neural plate, such as *tlc*, *fgf3*, and *fgf8*, mimic properties of the organizer activity produced by the first row of anterior neural plate cells in zebrafish, or the anterior neural ridge in other model vertebrates (Houart et al., 1998, 2002; Crossley et al., 2001; Echevarria et al., 2003; Walshe and Mason, 2003; Storm et al., 2006). In zebrafish, *emx3*, previously named *emx1* (Morita et al., 1995; Houart et al., 1998; Derobert et al., 2002; Kawahara and Dawid, 2002), is one of the earliest transcription factors expressed in response to anterior neural plate organizer activity. However, although *emx3* has been used extensively as a telencephalic marker, virtually nothing is known about its function.

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We used gene nomenclature approved by ZFIN (http://zfin.org/zf_info/nomen.html).

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Two paralogs of *emx3*, *emx1* and *emx2*, have been identified in all currently characterized vertebrate genomes; *emx3*, however, has only been found in a shark and several teleost genomes, not in the tetrapod lineage (Derobert et al., 2002; Kawahara and Dawid, 2002). Because sharks are ancestral to bony fish and tetrapods, it is possible that *emx3* was present in a common ancestor of fish and tetrapods and was subsequently lost in the tetrapod lineage (Derobert et al., 2002; Kawahara and Dawid, 2002).

In mice, Emx2 patterns the rostrocaudal axis of the cerebral cortex and is necessary for cortical radial layering (O'Leary et al., 2007). When Emx2 function is lost, positional information is shifted caudally and medially, and gene expression patterning and thalamocortical connectivity are affected. In addition, *Emx2* knockout mice have small olfactory bulbs and poorly differentiated cortical cell layers. Expression of *Emx1* and *Emx2* is highest in dorsomedial structures such as the hippocampus and dentate gyrus. In these regions, cortical defects are most pronounced when gene function is lost (Yoshida et al., 1997; Mallamaci et al., 2000a; Bishop et al., 2002, 2003; Shinozaki et al., 2004). The reduced hippocampal structures in *Emx2* knockout mice are thought to result from altered proliferation patterns (Tole et al., 2000; Heins et al., 2001; von Frowein et al., 2006). The role of *Emx1* is less apparent, because *Emx1* mutant mice have minor structural defects in the forebrain (Qiu et al., 1996; Yoshida et al., 1997). Homozygous mutant *Emx1* mice are viable and behavioral tests showed impaired motor learning and reduced learning-induced hippocampal neurogenesis in adult mutants (Hong et al., 2007).

Zebrafish *emx1* and *emx2* are expressed in largely overlapping domains in the telencephalon and in nonoverlapping domains in the diencephalon. *emx1* expression starts around the 17-somite stage (17.5 hr; hours postfertilization) in dorsal telencephalon and continues until after 24 hr (Kawahara and Dawid, 2002). *emx2* is expressed in ventral diencephalon at early somite stages and starts to be expressed throughout the dorsal telencephalon by the 17-somite stage, with the exception of a small domain in the dorsomedial telencephalon (Morita et al., 1995). At bud stage (10 hr), the horseshoe-shaped expression domain of zebrafish *emx3* (previously called *emx1*) and other genes at the anterior edge of the neural plate demarcate the prospective telencephalon (Wilson and Houart, 2004). Within this domain, *emx3* is expressed in a two- to six-cell-wide stripe (Morita et al., 1995; Houart et al., 1998). During somitogenesis, *emx3* mRNA is expressed in a gradient from high levels in dorsal telencephalon to low levels in ventral telencephalon and is absent from the most ventral third of the telencephalon (Morita et al., 1995). This expression pattern suggests a role of *emx3* in dorsoventral patterning of the telencephalon.

In this study, we analyzed the roles of *emx1*, *emx2*, and *emx3* in zebrafish forebrain development. We also identified an *Emx3* ortholog in the gray short-tailed opossum (*Monodelphis domestica*) and in *Xenopus tropicalis* genomes. This is the first report of tetrapod genomes that contain all three *emx* genes. Our results suggest that *emx3*, but not *emx1* or *emx2*, is necessary in a time- and dose-dependent manner for differentiation and axon tract formation of dorsal telencephalic neurons, and may involve activation of the Wnt/beta-catenin pathway. We find that the roles of zebrafish *emx* genes differ from those of their mouse orthologs.

RESULTS

Emx1, Emx2, and Emx3 Are Distinct Paralogs in Fish and Other Vertebrates

To identify paralogy and orthology relationships with Emx proteins in other species, we searched several genome sequence databases for previously unpublished sequences encoding Emx3. The stickleback (*Gasterosteus aculeatus*) and tetraodon (*Tetraodon nigroviridis*) genomes contain predicted full-length genes encoding Emx1, Emx2, and Emx3

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proteins. We also found three potential *emx3* exon sequences in the elephant shark (*Callorhinchus milii*) genome, version 1.4x (Venkatesh et al., 2007). Although these putative *emx3* exons do not lie on a contiguous stretch of DNA, they encode a putative full-length Emx3 protein with conserved intron positions and 10 of 11 amino acids unique to Emx3, compared with Emx1 and Emx2. Using translated BLAST (Altschul et al., 1997), we found a full-length Emx3 sequence encoded in the opossum (*Monodelphis domestica*) genome and a *Xenopus tropicalis* cDNA. The encoded proteins are 57% and 69% identical to dogfish Emx3, and contain 4 and 6 of 11 Emx3-specific residues (Derobert et al., 2002). The region between the amino terminal and homeodomain is most divergent from other Emx3 proteins. Both Emx1 and Emx2 are present in the *Xenopus tropicalis* genome. The opossum genome also contains a full-length predicted *Emx2* gene and a partial predicted *Emx1* exon 1 that groups with mammalian *Emx1* sequences in a neighbor-joining tree of translated exon 1 sequences (data not shown). The opossum partial *Emx1* exon1 sequence contains a frameshift and a stop codon, and may, therefore, be a pseudogene. These results suggest that both the opossum and *Xenopus tropicalis* genomes contain all three *Emx* paralogs, although the opossum *emx1* gene may no longer be functional.

We aligned these new predicted Emx3 sequences with representative Emx sequences encoded by full-length genes or expressed sequence tags to calculate a neighbor-joining phylogenetic tree (Fig. 1A). The tree confirms three distinct groups of predicted Emx protein sequences with high bootstrap values (Fig. 1A). In addition to overall sequence conservation within the group, the sequences share most amino acids that were previously identified as unique to the *Emx* paralog groups (Derobert et al., 2002).

To investigate orthology relationships of *emx1*, *emx2*, and *emx3* genes further and to analyze whether other tetrapod *Emx3* genes have been lost (Derobert et al., 2002; Kawahara and Dawid, 2002), we compared conserved synteny of *Emx* genes with neighboring genes in assembled fish and tetrapod genomes (Fig. 1B–D). We identified four genes within 0.3 Mb of zebrafish *emx1* and five genes within 1 Mb of zebrafish *emx2*, with shared synteny in tetrapod genomes. The orthologs of these genes were located within 3 Mb or less of the respective *Emx* ortholog on stickleback, tetraodon, chick, mouse, and human chromosomes (Fig. 1B,C). *Sfxn5* is an immediate neighbor of *Emx1* in all analyzed species including the opossum *Emx1* gene fragment. *Rab11fip2* is an immediate neighbor of *Emx2* in all species analyzed except zebrafish, where this gene is located on a different chromosome. Within 2 Mb of *emx3* in zebrafish, we found eight genes with conserved synteny in opossum and at least two other species including human, mouse, or chicken (Fig. 1D). Most of these genes are also close to *emx3* in stickleback and tetraodon. The genes *Emx3*, *Tmed9*, and *B4galt7* lie within a 50-kb region in all the genomes we analyzed. Thus, although *Emx3* appears to be missing from chicken, human, and mouse genomes, the synteny of the region around *emx1*, *emx2*, and *emx3* has been conserved throughout vertebrate genome evolution. These results are consistent with the hypothesis that *Emx3* was present in a common vertebrate ancestor and has either been lost from several tetrapod sublineages, or has not yet been discovered in human, mouse, or chicken.

Based on their low bootstrap values and short branch lengths, Emx1 and Emx3 may be more closely related to each other than to Emx2. This interpretation predicts that we should find more conserved syntenic relationships between *Emx3* and *Emx1* than between these genes and *Emx2*. To test this hypothesis, we examined conserved synteny among *Emx* genes from different groups. We found, for example, that *Nanos1* is syntenic with *Emx2* in tetrapods but syntenic with *emx1* in fish; stickleback has an additional *nanos1* gene adjacent to *emx3*. *smyd5* is closely linked to zebrafish and stickleback *emx3*, whereas the chicken, mouse, and human *Smyd5* orthologs are linked to *Emx1*. Thus, syntenic relationships with these markers

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do not support the conclusion that *Emx1* and *Emx3* are more closely related to each other than to *Emx2*.

Morpholino Knockdown of *emx3* and *emx1*, But Not *emx2*, Reduces Expression of Dorsal Telencephalic Genes

To complement previously published gene expression analyses (Morita et al., 1995; Houart et al., 1998; Kawahara and Dawid, 2002), we studied the mediolateral expression patterns of *emx1*, *emx2*, and *emx3* in the telencephalon (Fig. 2). *emx1* is expressed directly adjacent and dorsal to the olfactory organ, and is excluded from the ventricular zone. *emx2* and *emx3* are expressed throughout the dorsal telencephalon including the ventricular zone, with the strongest expression adjacent and dorsal to the olfactory organ. *emx2* and *emx3*, but not *emx1*, are also expressed in the ventral diencephalon (Fig. 2A–C).

We tested whether knock down of *emx1*, *emx2*, or *emx3* function with morpholino antisense oligonucleotides (MO) affects the regionalization and differentiation of the forebrain. We used a combination of two translation blocking morpholinos against each transcript because this combination resulted in the strongest phenotype for *emx3*MOs (Fig. 3D) and *emx1*MOs (Fig. 3B). To block morpholino toxicity (Robu et al., 2007), all experimental and control injections contained *tp53*MO (see the Experimental Procedures section). *emx*MO thus refers to injection of two translation blocking morpholinos and *tp53*MO unless otherwise indicated.

To assess the specificity of *emx3*MO, we injected single and combinations of *emx3*MO morpholinos and consistently obtained the same phenotypes (Fig. 3A,D,E). To assess the effectiveness of *emx3*MO, we identified two aberrantly spliced products by reverse transcriptase-polymerase chain reaction (RT-PCR) of embryos injected with *emx3* splice blocking morpholinos: an exon1–exon3 fusion (Fig. 3H) that introduces a frameshift leading to an early stop codon in exon3, and exon 3 spliced to the cryptic splice site within exon 2 (Fig. 3I) that produces an in-frame deletion leading to loss of the second helix of the homeodomain. Because at least the exon1–exon3 out of frame fusion is likely not functional, and we because obtain a phenotype with these *emx3* splice morpholinos, it is likely that this phenotype is due to knockdown of *emx3*. The ability to rescue *emx3*MO phenotypes with *emx3* mRNA that had been modified so it was not complementary to *emx3* translation blocking morpholinos (Fig. 6) provides further evidence that the observed *emx3*MO phenotypes are indeed due to knockdown of *emx3*. YFP expression in Tg(*emx3:YFP*)^{b1200} embryos is lost using translation blocking morpholinos but not with splice blocking morpholinos (data not shown), indicating that translation blocking morpholinos indeed knock down mRNA that is complementary to the MO sequence in its 5′ untranslated region. Together, these results suggest that the *emx3* morpholino phenotypes we observe are specific and due to knockdown of *emx3* function.

*emx1*MO-injected embryos showed a slight reduction of *eomesa* (*tbr2*; Fig. 3B), *tcf4l* (not shown), and *atoh2b* expression (not shown), whereas *emx2*MO injected embryos did not show changes in the expression patterns of *eomesa* (Fig. 3C) or any of the other genes tested for phenotypes with *emx3*MO. We confirmed the absence of phenotypes with an *emx2* splice blocking morpholino that abolished a large portion of correctly spliced mRNA as determined by RT-PCR (data not shown) and led to aberrant out of frame splicing of exon 1 to exon 3 (Fig. 3G). Apart from having little or no effect on marker gene expression, coinjection of *emx1*MO and *emx2*MO together with *emx3*MO did not enhance the *eomesa* expression defect (Fig. 3F), nor any of the *emx3*MO phenotypes that we describe below (Fig. 4). We suggest that *emx1* and *emx2* have subtle, or perhaps, later functions in forebrain development, or that the markers we used did not detect their roles.

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In embryos injected with *emx3*MO, several genes had smaller and less intense dorsal telencephalic expression domains than control embryos as tested by in situ hybridization (Fig. 4). These phenotypes were mostly subtle, but all of them were consistently present in >90% of *emx3*MO injected embryos (n > 18 embryos for each marker). Differences were apparent from the onset of expression of the respective genes in the telencephalon, which for most of the affected genes corresponds approximately to the 18-somite stage (18 hr). Affected markers include the proneural basic helix–loop–helix factor genes *tcf4l*, *atoh2b*, and *neurod* (Fig. 4A–C′), the *elavl4* (*HuD*) RNA binding protein (Fig. 4D–D′), and the vesicular glutamate transporters *slc17a6* and *slc17a6l* (Fig. 4G–H′). Their most strongly affected mRNA expression domains are located adjacent and dorsal to the olfactory placodes and may therefore include the prospective olfactory bulb, the region into which olfactory sensory axons project around 35 hr (Whitlock and Westerfield, 1998). Neuronal differentiation markers for telencephalic neurons that are more broadly expressed in the telencephalon, such as *elavl3* (*HuC*) and *reln* (Fig. 4E–F′), appear unaffected by *emx3*MO, although in some embryos, the region directly ventral to the presumptive future olfactory placode appears to be less labeled in some embryos (e.g., as in Fig. 4E,E′ close to the letter t). Similarly, the transcription factors *eomesa*, *dlx2a*, *lhx5*, *pax6a*, *emx1*, *fezf2* (Fig. 4I–N′), and *foxp2*, are reduced only in their dorsal telencephalic expression domain, but not in ventral telencephalon or diencephalon. *emx2*, *emx3*, and the zinc finger transcription factor, *fezf2* (Fig. 4M–M′), are reduced only in the region of strong expression adjacent and dorsal to the olfactory placode, whereas the weaker expression domain that spans the dorsal telencephalon is normal. We observed no changes in expression for genes expressed in ventral telencephalon, such as *fzd8a* (Fig. 4N–N′), *isl1*, *sfrp5*, and the GABAergic neuronal markers *gad1* and *gad2* (not shown). Markers expressed in the most dorsal part of the telencephalon, such as *wnt8b*, *lef1*, and *axin2*, are also unaffected by *emx3* morpholinos, as are the Wnt7b duplicates *wnt7ba* and *wnt7bb* (Fig. 4O–P′, and data not shown) that are expressed along the dorsal part of the telencephalic–diencephalic border. The fibroblast growth factor (FGF) pathway components with broad telencephalic expression patterns such as *fgf8a* (Fig. 4Q–Q′), *spry4*, and *pea3* (not shown) are equally unaltered, as is *foxg1* (*bf1*, Fig. 4R,R′) that is expressed throughout the telencephalon except the most dorsal tip. These unaffected gene expression domains show that the size of the telencephalon and the shapes of cells are normal at 24 hr in *emx3*MO-injected embryos. From these results, we conclude that *emx3* knockdown impairs specification or differentiation of dorsal telencephalic neurons. However, the dorsal telencephalon, as such, is still specified, and loss of *emx3* does not appear to affect specification of dorsal vs. ventral telencephalon.

Knockdown of *emx3*, but Not *emx1* or *emx2*, Causes Axon Outgrowth and Fasciculation Defects

After the 18-somite stage (18 hr), when most changes in marker gene expression become apparent in *emx3*MO injected embryos, telencephalic neurons extend axons through the supraoptic tract (SOT) into the diencephalon and then, later, across the ventral telencephalon, thereby forming the anterior commissure (AC; Chitnis and Kuwada, 1990; Wilson et al., 1990; Ross et al., 1992). To test for defects in axon formation or pathfinding, we labeled axons with antibodies against acetylated tubulin. By 35 hr, when 92% (n = 39) of control embryos had developed a well-formed AC, 53% (n = 51) of *emx3*MO-injected embryos had extended only five or fewer axons across the AC (Fig. 5A–D). The SOT of controls developed into a wide band of axons across the telencephalic surface with one slightly separate fascicle at its anterior end. Although the SOT was present after *emx3* knockdown, it was thinner and split into two to four irregular fascicles (53%, n = 32; Fig. 5A,B). In addition, the olfactory nerve was defasciculated at its entry point into the telencephalon (Fig. 5C,D), where *emx3* expression was also previously reported (Whitlock and Westerfield, 2000). At 2 and 3 days of development, *emx3*MO injected embryos had

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fewer presumptive olfactory glomeruli that we labeled with by Bodipy ceramide (Shanmugalingam et al., 2000), indicating that the olfactory bulb was not differentiating normally (Fig. 5E–F). Knockdown of *emx1* and *emx2* had no observable effects on axon tracts (not shown).

Cell Proliferation Patterns Are Relatively Unaffected in *emx3* Morpholino Injected Embryos

We tested whether cell proliferation defects may underlie the reduced number of differentiated dorsal telencephalic cells after *emx3* knockdown. For example, progenitor cells may remain longer in a proliferative state instead of differentiating, potentially producing more proliferative cells or proliferation in ectopic locations. Alternatively, progenitor cells may fail to divide in the absence of *emx3*, producing fewer differentiated cells. To test whether cells keep proliferating instead of differentiating, or are dividing at ectopic locations at the time we find reduced marker gene expression, we examined *emx3*MO-injected embryos for changes in the quantity or position of mitotic cells using 5-bromo-2-deoxyuridine (BrdU) to label S-phase nuclei, and antibodies against Ser10-phosphorylated histone H3 (pH3) to label the condensed chromatin of mitotic cells. At 24 hr, the cells that incorporate BrdU during a 5-min incubation period are located in a three- to four-cell-wide area close to the ventricular zone in control and *emx3* knockdown embryos; the ventricular zone is slightly expanded in some *emx3*MO injected embryos (Fig. 5G–H). Mitotic cells labeled by pH3 are located in the ventricular zone of the telencephalon, closer to the ventricular surface than BrdU-labeled S-phase cells, in both control and *emx3*MO injected embryos (Fig. 5G,H). We counted mitotic cells in the telencephalon in embryos double-labeled with pH3 and fluorescent in situ hybridization for *foxg1* and *emx3* mRNA to outline the telencephalon. We found no difference in the number of pH3-positive cells in a 54-μm-wide confocal stack in the dorsal or ventral halves of the telencephalon in embryos injected with *emx3*MO (27 ± 6 cells, 57 ± 6% of total pH3 cells, n = 33 embryos) compared with control embryos (26 ± 5 cells, 61 ± 8% of total, n = 31 embryos) (Fig. 5I–K). The stacks used for counting included all cells in the central ventricle between the left and right halves of the telencephalon. *emx3* morpholino injection did not affect the uniform distribution of cells labeled by pH3 in the ventricular zone or at the telencephalon–diencephalon boundary (Fig. 5I,J). These data suggest that knockdown of *emx3* has a very small, if any influence on cell division at the stage when gene expression patterns are most affected. Therefore, even though loss of *emx3* function keeps cells from differentiating, it does not keep cells in a proliferative state, nor does it cause cells to proliferate at ectopic locations.

emx3 mRNA Is Required Before the Five-Somite Stage to Rescue *emx3*MO Phenotypes

To rescue the *emx3* morpholino phenotypes without disrupting early development, we conditionally activated green fluorescent protein (GFP)-tagged *emx3* mRNA in heterozygous *Tg(hsp70l:emx3-myc-GFP)*^{b1202} transgenic embryos that had been injected with *emx3*MO. A 10-min heat shock at the one-somite or two-somite stage (10.5 hr), shortly after the onset of endogenous *emx3* expression at bud stage (10 hr), that results in weak GFP fluorescence throughout the embryo in transgenic siblings, at least partially restores expression of *tcf4l*, *HuD*, and *eomesa* in 86–95% of embryos (n > 7 for each marker), compared with nontransgenic siblings that did not express GFP, without any apparent defects (Fig. 6 and data not shown). Heat shock of 30 min gave rise to embryos with small eyes and ventrally expanded markers of dorsal telencephalon (Fig. 6A,H), as described for overactivation of the canonical Wnt/beta-catenin pathway (van de Water et al., 2001). This phenotype is different from the loss of forebrain and eyes that is most frequently observed upon overactivation of the Wnt/beta-catenin pathway (Stachel et al., 1993;Kelly et al., 1995;Heisenberg et al., 2001;van de Water et al., 2001;Kim et al., 2002). We did not observe rescue of anterior commissure defects; however, anterior commissure was also lost

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in control embryos upon strong activation of the transgene, as indicated by high levels of GFP expression. This presumably indicates that axon outgrowth is too dose sensitive for this method of rescue. For embryos heat shocked at the five-somite stage (11.5 hr) or later, we observed neither rescue nor overexpression phenotypes (Fig. 6A,D). Heat shock at bud stage failed to induce uniform GFP expression (not shown). Our results indicate that *emx3* is required before the five-somite stage for the expression of dorsal telencephalic markers, and that timing and dosage of Emx3 protein are critical requirements for normal function.

emx1, *emx2*, or *emx3* Overexpression Dorsalizes the Embryo and Posteriorizes the Central Nervous System

We tested whether gain of *emx1*, *emx2*, or *emx3* function affects marker gene expression or axon phenotypes (Fig. 7). Injection of 12–25 pg of *emx1*, *emx2*, or *emx3* mRNA per embryo at the one-cell stage strongly dorsalizes and posteriorizes embryos in a concentration-dependent manner. At bud stage, injected embryos are severely egg-shaped (Fig. 7E,M), the notochord is broadened as seen by *flh* expression (Fig. 7E), the forebrain and eye field markers *pax6b* (Fig. 7G) and *rx3* (not shown) are reduced to a small spot at the animal pole or lost completely, and the posterior central nervous system (CNS) marker *egr2b* (*krox20*) is shifted anteriorly (Fig. 7G). At 35 hr, embryos lack eyes and have curled tails (Fig. 7B,C), and the gene expression domains of *otx2* (Fig. 7I) and *foxb1.2* (*mar*, not shown) are shifted anteriorly. All of these phenotypes are identical to those seen after activation of the Wnt/beta-catenin signaling pathway by LiCl treatment, after *wnt* mRNA injection, or in *masterblind* (*axin1*) mutant embryos (Fig. 7A–C,H; Stachel et al., 1993;Kelly et al., 1995;Heisenberg et al., 2001;van de Water et al., 2001;Kim et al., 2002). The Wnt/beta-catenin pathway inhibitor *dkk1* (Shinya et al., 2000) and the Fgf pathway inhibitor *spry4* (Fürthauer et al., 2001) both rescue loss of eyes and curled tails in *emx1*, *emx2*, *emx3*, and *wnt8b* mRNA injected embryos (Fig. 7H). These results suggest that ectopic *emx* mRNAs and *Wnt/beta-catenin* signaling affect the same mechanisms that regulate early dorsal–ventral patterning of the embryo and anterior–posterior development of the central nervous system. The rescue of this phenotype with an FGF inhibitor is expected because many aspects of beta-catenin–dependent dorsalization are mediated by activation of the FGF signaling pathway (Maegawa et al., 2006). Embryos injected with *emx* mRNA, however, do not resemble control embryos injected with *fgf8* mRNA that are not egg-shaped at bud stage, do not lose eyes, and always have a deformed tail. Consistent with our finding that emx morpholino injection does not affect expression of *fgf8*, nor expression of the FGF downstream genes *spry4* and *pea3* during somitogenesis or at 24 hr, we consider it unlikely that *emx3* is involved in activating FGF signaling. The effect of *emx* mRNA overexpression is probably not related to the endogenous function of *emx* in the early embryo because none of the *emx* genes are expressed before the end of gastrulation (data not shown). Nevertheless, the similarity of overexpression phenotypes both by heat shock and early mRNA expression raise the possibility that *emx* gene function during development of the telencephalon also involves Wnt/beta-catenin pathway activation.

We used the mRNA overexpression phenotypes to investigate whether Emx3 acts as a transcriptional activator or repressor. We injected mRNA encoding a fusion of the Emx3 homeodomain (*emx3hd*) with either the transcriptional activator domain of VP16 or the transcriptional repressor domain of *Drosophila engrailed* (*en*). The *emx3hd-en* fusion mRNA gave rise to the same phenotypes as *emx3* mRNA, including egg-shaped embryos at bud stage (Fig. 7L,M), whereas embryos that injected with the *emx3hd-vp16* fusion did not resemble embryos with overactivated Wnt/beta-catenin pathway at any stage, nor did they resemble *emx3*MO embryos (Fig. 7K and data not shown). These data suggest that *emx3* acts as a transcriptional repressor, but do not exclude a different mode of action in the telencephalon.

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Emx3 Regulates Telencephalic Gene Expression Cell-Autonomously

Our analysis of *emx3* mRNA overexpression indicated that *emx3* could possibly play a role in cell signaling. *emx3* might enhance signaling in the telencephalon in cells that secrete or receive signals, either cell-autonomously or non–cell-autonomously. We tested for a possible non–cell-autonomous role of *emx3* by transplanting wild-type cells labeled with Alexa 555 dextran into *emx3*MO injected host embryos at blastula stages and analyzed the resulting chimeric embryos for *atoh2b* expression at 24 hr. We found that only uninjected donor cells express *atoh2b* mRNA when transplanted into the normal *atoh2b* expression domain, whereas cells derived from *emx3*MO injected host embryos never express *atoh2b*, even when immediately adjacent to the transplanted wild-type cells in the telencephalon or the olfactory placode (n = 7 embryos; Fig. 8A,B). In addition, mosaic, heat shock-induced over-expression of Emx3-myc-GFP in embryos injected with *hsp70l:emx3-myc-GFP* plasmid DNA induced the expression of *emx1* cell-autonomously (Fig. 8C). Thus, we conclude that *emx3* acts cell-autonomously in telencephalic cells. We suggest that, if *emx3* activates a signaling pathway, it does so in signal receiving cells.

DISCUSSION

The *Emx3* Gene Was Lost During Expansion of the Tetrapod Lineage

Our phylogenetic analysis of Emx protein sequences and shared syntenies confirms that the vertebrate *Emx1*, *Emx2*, and *Emx3* genes constitute different paralog groups rather than local tandem duplicates. In addition, we identified all three *Emx* genes in *Xenopus tropicalis* and in the opossum (*Monodelphis domestica*), a marsupial mammal. In other vertebrate genomes that lack the *Emx3* ortholog, we identified syntenic regions similar to opossum, suggesting that Emx3 was lost in these species. Opossum and *Xenopus Emx3* provide the first evidence for *Emx3* genes in tetrapods and rule out the possibility that *emx3* was lost from the tetrapod lineage (Derobert et al., 2002; Kawahara and Dawid, 2002). We suggest that *Emx3* is an ancient gene that was lost in some, and preserved in other vertebrate sublineages.

emx3 Is Required Early During Neurulation to Regulate Neural Differentiation in the Developing Telencephalon

We analyzed *emx3* gene function by morpholino knockdown. Our results indicate that *emx3* functions specifically in the differentiation of dorsal telencephalic neurons. Expression of many different types of genes in this region, such as transcription factors, neurogenic genes, and neurotransmitter transporters, are reduced by *emx3* knockdown (Figs. 2, 3). These cells probably include but may not be limited to neurons of the prospective olfactory bulb, the region adjacent and dorsal to the olfactory placode into which the olfactory nerve axons later extend (Whitlock and Westerfield, 1998). Some of the affected genes have additional expression domains in ventral telencephalon or other areas of the brain, and these appear normal in *emx3*MO-injected embryos. Thus, the effects of *emx3* seem to be specific to dorsal telencephalic development.

However, expression of most markers is not completely abolished, and *emx3*MO injection affects broadly expressed neuronal differentiation markers such as *elavl3* and *reln* only to a small degree. These observations indicate that either the affected neurons constitute a small fraction of dorsal telencephalic neurons or that the affected neurons differentiate to some extent in *emx3*MO-injected embryos. We find no ectopic proliferation outside the ventricular zone, which indicates that the undifferentiated cells have exited the cell cycle. We therefore conclude that the major role of *emx3* is to activate the expression of genes that are specific to the dorsal telencephalon. In accordance with this interpretation, we were able to activate expression of *emx1* ectopically in cells expressing GFP-tagged *emx3*. The axonal

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defects and failure to form glomeruli are presumably consequences of aberrant and incomplete neural differentiation.

emx3 is one of the first genes expressed in the neural plate that demarcates prospective dorsal–ventral patterning of the telencephalon. However, phenotypes resulting from *emx3* knockdown do not appear until the 18-somite stage (18 hr), whereas rescue of *emx3*MO injection phenotypes requires expression of *emx3* mRNA at the one- or two-somite stage (10.5 hr). Because we cannot exclude the possibility that residual *emx3* function from incomplete knockdown masks possible early phenotypes, or that earlier phenotypes are very subtle, it is possible that the late phenotypes are secondary consequences of earlier, undetected functions of Emx3. There are, however, other examples of genes expressed early that apparently function only later in development. *fezf2*, for example, starts to be expressed even earlier than *emx3* in prospective telencephalon, and both mutant and morpholino injected animals lack specific subsets of monoaminergic neurons without displaying early patterning defects (Levkowitz et al., 2003; Jeong et al., 2007).

In this study, knockdown of *emx3* alone produced a strong phenotype. We found a slight reduction in expression of few dorsal telencephalic differentiation markers in *emx1*MO-injected embryos, and we did not observe any phenotypes resulting from *emx2* knockdown. We also found no evidence of synergy when knocking down multiple *emx* genes. It is possible that *emx1* and *emx2* morpholinos failed to knock down *emx1* and *emx2* translation sufficiently enough to produce a phenotype. Alternatively, *emx1* and *emx2* gene functions may be more subtle or required later in development.

Because zebrafish *emx1* and *emx2* play no obvious roles in patterning, it is possible that zebrafish *emx3* supplies the functions provided by *Emx1* and *Emx2* in mouse. The reported mouse *Emx* knockout mutations affect the olfactory bulb and cortex, which correspond to the zebrafish pallium, the dorsal telencephalon (Wullimann and Mueller, 2004). In both mouse and fish, *Emx* loss-of-function affects only pallial structures, as indicated by altered gene expression patterns (Qiu et al., 1996; Yoshida et al., 1997; Guo et al., 2000; Mallamaci et al., 2000b; Bishop et al., 2002, 2003; Shinozaki et al., 2004). In *Emx2* knockout mice, the BrdU-incorporating ventricular zone of the cortex is broader, indicating a more immature cortex (Mallamaci et al., 2000b; Tole et al., 2000; Bishop et al., 2003). Similarly, we find that knockdown of zebrafish *emx3* produces a slight expansion of the ventricular zone and adjacent BrdU-positive layer, similar to the mouse *Emx2* knockout phenotype. On the other hand, however, we find major differences between mouse *Emx2* and zebrafish *emx3* functions in regulating expression of other genes. *fgf8* expression in zebrafish telencephalon is not affected by *emx3* knockdown, whereas loss and gain of function studies of mouse *Emx2* suggest that Emx2 represses Fgf8 (Fukuchi-Shimogori and Grove, 2003; Hamasaki et al., 2004). Similarly, dorsal *Emx2* and ventral *Pax6* repress each other in the mouse cortex (Muzio et al., 2002), whereas we find that *emx3* positively regulates *pax6a* expression, although it is likely that the zebrafish *pax6a* expression domain in the telencephalon does not correspond to the *Pax6* domain in the mouse cortex (Wullimann and Mueller, 2004). Mouse *Emx2* positively regulates *reelin* expression (Mallamaci et al., 2000a), whereas we do not find zebrafish *reln* regulated by *emx3*. These comparisons suggest that mouse *Emx* genes may function in a genetic network that arose as an innovation during evolution of the mammalian cortex.

emx3 May Play an Activating Role in the Wnt/beta-catenin Pathway

To rescue *emx3*MO induced phenotypes, *emx3* mRNA needed to be expressed at low levels at the 1-somite or 2-somite stage (10.5 hr). Heat shock induced expression at higher levels, or only slightly earlier at bud stage, led to a marked ventral expansion of dorsal telencephalic markers and concomitant loss of eyes. This phenotype has been reported as a

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less frequent effect of Wnt/beta-catenin overactivation (van de Water et al., 2001) that is distinct from the usually observed loss of both forebrain and eyes (Stachel et al., 1993; Kelly et al., 1995; Heisenberg et al., 2001; van de Water et al., 2001; Kim et al., 2002). Our consistent observation of this otherwise rare phenotype may point to an interesting, time-sensitive effect of Wnt/beta-catenin signaling in which *emx3* may play a role. Alternatively, this phenotype may be independent of Wnt/beta-catenin signaling and may reflect an ability of *emx3* to expand prospective dorsal telencephalon at the expense of prospective eye field independently of Wnt/beta-catenin signaling. Currently, we cannot distinguish between these possibilities. In either case, sensitivity to overexpression ends around the five-somite stage (11.5 hr). *emx3* function thus requires specific expression levels and timing.

In accordance with an involvement of the Wnt/beta-catenin pathway in Emx function, *emx1*, *emx2*, or *emx3* overexpression phenotypes induced by injection of mRNA at the one-cell stage are similar to overactivation of the canonical Wnt pathway (Stachel et al., 1993; Kelly et al., 1995; Heisenberg et al., 2001; van de Water et al., 2001; Kim et al., 2002). The overexpression phenotypes are indistinguishable from the effects of *wnt8b* mRNA overexpression. Because the homeodomain-engrailed fusion, but not the homeodomain-VP16 fusion, phenocopies *emx3* mRNA, *emx3* may act as a transcriptional repressor, at least in this context.

Additional information is needed to link the physiological role of *emx3* in telencephalic development conclusively to Wnt/beta-catenin signaling, for example, by identifying a Wnt/beta-catenin downstream target in the telencephalon that also requires *emx3* function. In mice, Emx2 regulates many components of the canonical Wnt/beta-catenin pathway, including *Wnt7b*, *Wnt8b*, and *Axin2* (Muzio et al., 2002; Machon et al., 2007). In contrast, we find no change of *wnt7ba*, *wnt7bb*, *wnt8b*, or *axin2* expression after *emx* knockdown. The fact that the *emx3* overexpression phenotypes can be rescued by an extracellular inhibitor of the Wnt/beta catenin pathway (*dkk1*) is consistent with a model in which *emx3* activates expression of an extracellular Wnt ligand. However, rescue by an extracellular inhibitor does not exclude an alternative model in which *emx3* activates the Wnt pathway internally in signal receiving cells, for example (but not necessarily) as an activating transcriptional cofactor of the TCF/LEF transcriptional complex. In the latter case, *emx3* would be expected to act cell-autonomously, as opposed to a non–cell-autonomous function if *emx3* activates a secreted ligand. Our overexpression experiments in chimeric embryos suggest that *emx3* acts indeed cell-autonomously, at least with respect to activating expression of *emx1* and *atoh2b*. These results favor a model in which *emx3* acts in signal receiving cells, but is not strictly required for Wnt/beta-catenin pathway activation (otherwise rescue with *dkk1* would not be possible) but rather plays an enhancing or balancing role that can be easily overridden. Both *wnt7ba* and *wnt7bb* duplicates, expressed at the telencephalic-diencephalic border, and *wnt8b*, expressed at the caudal tip of telencephalon, are possible sources of a Wnt/beta-catenin signal that may function in dorsal telencephalic development.

Early in development, Wnt/beta-catenin signaling blocks forebrain development. Wnt signaling must be inhibited locally for forebrain development to occur properly and for *emx3* to be expressed (Wilson and Houart, 2004). A potential role of *emx3* in activating the Wnt/beta-catenin pathway may be to support differentiation of dorsal telencephalic neurons at the precise time when inhibiting Wnt/beta-catenin signaling may no longer be necessary for telencephalic specification. In mouse, Emx2 and Wnt/beta-catenin signaling regulate cortical neurogenesis in two ways. First, Emx2-mediated Wnt/beta-catenin pathway activation maintains cell proliferation that generates cells for the caudal–medial cortex (Muzio et al., 2005). Second, down-regulation of Wnt/beta-catenin signaling is necessary for neurogenesis (Hirabayashi et al., 2004; Machon et al., 2007). Establishing the link between *emx* genes and

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the Wnt/beta-catenin pathway, or other signaling pathways, will be important to elucidate the precise role of *Emx* genes in telencephalic neurogenesis.

EXPERIMENTAL PROCEDURES

Fish Maintenance and Transgenic Lines

AB or AB/TL hybrid zebrafish were raised under standard conditions at 28.5°C (Westerfield, 2007). Developmental stages were determined by morphological criteria or hr postfertilization (hr; Kimmel et al., 1995). Plasmid DNA injections for mosaic expression and generation of transgenic lines *Tg(emx3: YFP)*^{b1200} and *Tg(hsp70l:emx3-myc-GFP)*^{b1202} were performed by coinjection of I-*Sce*I enzyme (Roche, Basel, Switzerland, buffer from NEB, Ipswich, MA; Thermes et al., 2002) using a modified pG1 vector (a gift from Chi-Bin Chien) containing I-*Sce*I sites (introduced by H.-G. Belting).

Phylogenetic Analysis and Synteny Relationships

Alignments of full-length Emx protein sequences and phylogenetic trees were generated with ClustalX 2.0.3 (Larkin et al., 2007) (default parameter settings, positions with gaps not excluded, correction for multiple substitutions), and displayed using njplot 2.0 (Perriere et al., 1996). Genbank accession numbers, Ensembl/Genoscope/IMCB peptide ID, and species are *Callorhinchus milii* (elephant shark) Emx3 AAVX01298069.1:565-213 (putative exon 1), AAVX01519513.1:481-296 (putative exon 2), AAVX01149546.1: 535-699 (putative exon 3); *Danio rerio* (zebrafish) Emx1 AF534523, Emx2 D32215, Emx3 NM_131279; *Gallus gallus* (chicken) Emx1 ENS-GALP00000025888, Emx2 XM_421783; *Gasterosteus aculeatus* (stickleback) Emx1 ENSGACP00000006428, Emx2 ENSGACP00000004194, Emx3 ENS-GACP00000021610; *Homo sapiens* (human) Emx1 ENSP00000377670, Emx2 AF301598; *Lampetra japonica* (lamprey) Emx AB048758; *Monodelphis domestica* (South American opossum) Emx1 translated putative exon 1 fragment NW_001581849.1, bases 216282-216090, Emx2 ENSMODP0000-0011730, Emx3 XP_001381098; *Mus musculus* (mouse) Emx1 NM_010131, Emx2 AY117415; *Oryzias latipes* (medaka) Emx2 ENSORLP0000-0017758; *Scyliorhinus canicula* (dog-fish) Emx1 AF306637, Emx2 AF306636, Emx3 AF306635; *Takifugu rubripes* (fugu) Emx1 SINFRUP0-0000179547, Emx2 SINFRUP0-0000139710, Emx3 SINFRUP0000-0144814; *Tetraodon nigroviridis* (tetraodon) Emx1 GSTENP00019961001, Emx2 GSTENP00004254001, Emx3 GSTENP00024283001 (additional two predicted 5′ exons ignored); *Xenopus laevis* Emx1 NM_001093430; *Xenopus tropicalis* Emx1 NM_001005459, Emx2 ENSXETP00000047776, Emx3 CU075359 bases 567-1205 translated (Hubbard et al., 2007; Venkatesh et al., 2007). Conserved synteny was analyzed on http://www.ensembl.org/, using current database releases. Genes were considered orthologs if they were listed in Ortholog Predictions on the Ensembl Gene Report.

mRNA and Morpholino Injections, Heat Shock, and Cell Transplantation

Full-length *emx1* cDNA in pCS2+ was a gift from the Igor Dawid lab (Kawahara and Dawid, 2002). Full-length *emx2* cDNA, a gift from the Mishina lab (Morita et al., 1995), was subcloned into pCS2+ expression vector (Dave Turner and Ralph Rupp, unpublished observations). Full-length *emx3* cDNA was amplified by RT-PCR from mixed somite stage total RNA, sequenced for correctness, and ligated into a pG1-derived vector containing a myc tag followed by GFP. The *myc* tag serves as a spacer between *emx3* and *GFP* to preserve the functions of both proteins. Primers (ggatccaccatgtttcaacataacaaaaaatgcttcacgattgaatctcttgtg, ctcgagagatctgtcttctgaaatgacgtcaatgtcc) introduced six, silent, point mutations that reduced the sequence complementary to *emx3*MOatg translation blocking morpholino to a block of five bases and six blocks of two bases. For rescue experiments, embryos from crosses of AB

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and heterozygous *Tg(hsp70l:emx3-myc-GFP)*^{b1202} transgenic animals were injected with emx3MO, heat shocked at the desired stage for 10 min at 37°C, selected for presence or absence of *Emx3*-Myc-GFP fusion protein by fluorescence, raised to the desired stage at 28.5°C, and fixed for in situ hybridization or antibody labeling. A 10-min heat shock produced mRNA levels comparable to endogenous *emx3*, as determined by in situ hybridization with *emx3* probe in embryos fixed directly after heat shock. *emx3Homeodomain* (*emx3hd*)-*vp16* and *emx3hd-engrailed* (*en*) fusions were constructed by amplifying the *emx3* homeobox with primers including an artificial translation start consensus sequence (gcggatccaccatgcctttctcccgcaagcccaag, gactcgagctgcgggtctggagactcttcctc), and ligating to pCS2+ vector containing amplified PCR products of the VP16 transactivation domain (primers cgctcgaggcccccccgaccgatgtcagcctgg, gctctagactagtcccaccgtactcgtcaattc) and engrailed repressor domain (primers ctcgaggccctggaggatcgctgcagcccac, tctagatgcatagatcccagagcagatttc). mRNA transcription reactions (mMessage mMachine, Ambion, Austin, TX) were purified directly over RNeasy columns (Quiagen, Valencia, CA), eluted with water, and injected.

We used a combination of two translation blocking morpholinos (Gene Tools, Philomath, OR) against each gene (*emx1*MOatg, ccgttgccgagaacattgtccgtga; *emx1*MOupstream, cggtatgaccagaagagtccagct; *emx2*MOatg, acctcttcggtgtgggttgaaacat; *emx2*MOupstream, gtttaccagtcaagattccccagtt; *emx3MOatg*, cacttcttattgtgctggaacattg; *emx3*MO-upstream, gtcactctgaacccgatgatggag), a combination of two splice blocking morpholinos against *emx3* (*emx3*MOe2i2, gacgtgtctgtctcttacctgcgtc; *emx3*MOi1e2, tgtcgtcacctgcagagaaacaaac) at 0.05 pmol/embryo, or a splice blocking morpholino against *emx2* (*emx2*MOi1e2, gtaaacacattcttacctgagtttc) at 1 pmol/embryo. All morpholinos except *emx2*MOi1e2 induced ectopic cell death at concentrations that led to a phenotype and were therefore coinjected with *tp53*MO morpholino (ZDB-MRPHLNO-070126-7, gcgccattgctttgcaagaattg) (Robu et al., 2007) at 0.5 pmol per embryo in all experiments reported here. Approximately 10% of these embryos still showed low levels of ectopic cell death in the hindbrain, as judged by acridine orange staining (Shepard et al., 2004). Control embryos were injected with *tp53*MO alone.

For transplantation at blastula stages, donor oocytes were injected with 2% Alexa-555 10-kD dextran (Molecular Probes, D-22910, Eugene, OR). For fluorescent membrane labeling (Köster and Fraser, 2004), embryo medium contained 2 μg/ml Bodipy FL C5-ceramide (Molecular Probes, D-3521, Eugene, OR).

In Situ Hybridization, Antibody Labeling, and Cell Counting

Whole-mount in situ hybridization and antibody labeling were performed as described (Westerfield, 2007). Plasmids for probe synthesis were gifts from the respective authors, prepared by RT-PCR from embryonic total RNA, or purchased from ATCC (Manassas, VA): *atoh2b*, ZDB-GENE-010608-2 (Liao et al., 1999); *axin2*, ZDB-GENE-000403-2 (Peng and Westerfield, 2006); *dlx2a*, ZDB-GENE-980526-212 (Akimenko et al., 1994); *elavl3*, ZDB-GENE-980526-76 (Good, 1995); *elavl4*, ZDB-GENE-990415-246 (Thisse and Thisse, 2005); *emx1*, ZDB-GENE-031007-7 (Kawahara and Dawid, 2002); *emx2*, ZDB-GENE-990415-54 (Morita et al., 1995); *emx3*, ZDB-GENE-990415-53 (Morita et al., 1995); *eomesa*, ZDB-GENE-001228-1 (Mione et al., 2001); *egr2b*, ZDB-GENE-980526-283 (Oxtoby and Jowett, 1993); *fezf2*, ZDB-GENE-001103-3 (Levkowitz et al., 2003); *fgf8a*, ZDB-GENE-990415-72 (Fürthauer et al., 1997); *flh*, ZDB-GENE-990415-75 (Talbot et al., 1995); *foxb1.2*, ZDB-GENE-990616-47 (Odenthal and Nüsslein-Volhard, 1998); *foxg1*, ZDB-GENE-990415-267 (Toresson et al., 1998); *foxp2*, ZDB-GENE-041203-2 (Bonkowsky and Chien, 2005; Shah et al., 2006); *fzd8a*, ZDB-GENE-000328-3 (Kim et al., 1998); *gad1*, ZDB-GENE-030909-3 (Higashijima et al., 2004); *gad2*, ZDB-GENE-030909-9 (Higashijima et al., 2004); *isl1*, ZDB-GENE-980526-112 (Inoue et al.,

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1994); *lef1*, ZDB-GENE-990714-26 (Dorsky et al., 1999); *lhx5*, ZDB-GENE-980526-484 (Toyama et al., 1995); *neurod*, ZDB-GENE-990415-172 (Liao et al., 1999); *otx2*, ZDB-GENE-980526-406 (Li et al., 1994); *pax6a*, ZDB-GENE-990415-200 (Nornes et al., 1998); *pax6b*, ZDB-GENE-001031-1 (Nornes et al., 1998); *pea3*, ZDB-GENE-990415-71 (Münchberg et al., 1999); *reln*, ZDB-GENE-020822-1 (Costagli et al., 2002); *rx3*, ZDB-GENE-990415-238 (Chuang et al., 1999); *sfrp5*, ZDB-GENE-011108-2 (Peng and Westerfield, 2006); *slc17a6*, ZDB-GENE-030616-554 (Higashijima et al., 2004); *slc17a6l*, ZDB-GENE-050105-4 (Higashijima et al., 2004); *spry4*, ZDB-GENE-010803-2 (Fürthauer et al., 2001); *tcf4l*, ZDB-GENE-070829-1 (Brockschmidt et al., 2007); *wnt7ba*, ZDB-GENE-041210-178 (Carl et al., 2007); *wnt7bb*, ZDB-GENE-081006-1, accession no. FJ356091, cloned by RT-PCR (primers tggtggctctcggtgcgaacatcatc, tttgcaggtaaacacctccgtcctctc) to amplify partial predicted cDNA of *wnt7b* paralog ENSDARG00000071107; *wnt8b*, ZDB-GENE-990415-279 (Kelly et al., 1995).

Embryos were incubated with 10 mM BrdU (Roche 10280879001, Basel, Switzerland) for 10 min on ice, washed for 5 min, fixed for 2 hr, and labeled with mouse anti-BrdU antibody (Roche 11170376001, 1:100; Shepard et al., 2004). Antibody labeling was performed as described (Westerfield, 2007) using mouse anti–N-acetylated tubulin (Sigma T-6793, St. Louis, MO) 1:500; anti-pH3 rabbit polyclonal IgG (Upstate 06-570, Billerica, MA) 1:500; mouse anti-GFP (JL-8, BD Biosciences, Franklin Lakes, NJ) 1:500; Alexa 488- and Alexa 546-linked secondary antibodies (Molecular Probes, Eugene, OR) at 1:1,000. pH3 cell counts were performed on consecutive stacks of confocal sections from a Pascal confocal microscope (Zeiss, Wetzlar, Germany) using the Cell-Counter plugin of ImageJ (http://rsbweb.nih.gov/ij/).

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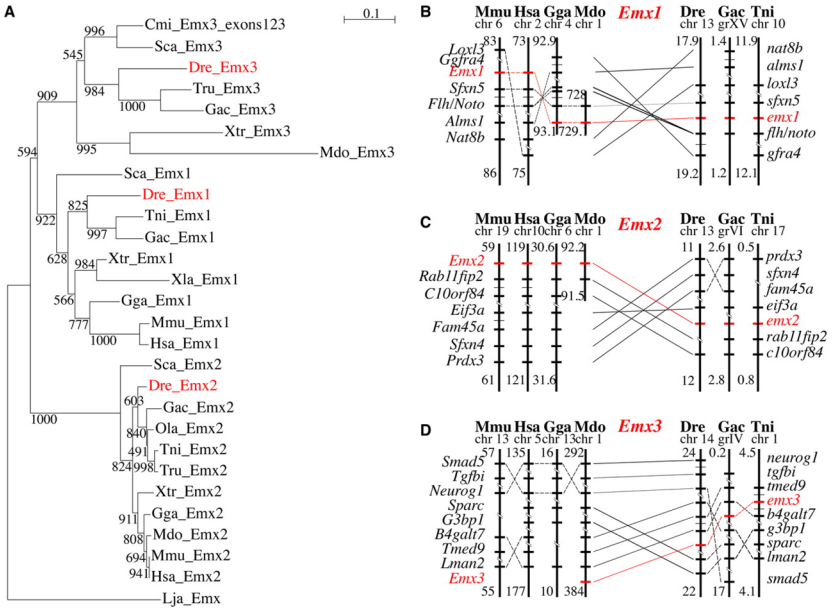

Fig. 1.

The *Emx3* gene is present in both fish and some tetrapod genomes. **A:** Phylogenetic tree of Emx protein sequences translated from cDNA or predicted sequences, calculated using the neighbor-joining method. Lamprey Emx is used as an outgroup. Branch lengths indicate the estimated number of amino acid substitutions per site; scale bar, 0.1 substitutions per site. Bootstrap values generating each node out of 1,000 replica are indicated. Cmi_Emx3_exons123 is complete but not on a contiguous stretch of DNA. **B–D:** Genomic analysis of *Emx* loci in several vertebrates. Conserved synteny of *Emx* genes confirms orthology relationship inferred from phylogenetic analysis. Syntenic regions containing *Emx1* (B), *Emx2* (C), and *Emx3* (D) are conserved among fish and tetrapods that contain them; in tetrapods lacking *Emx3* (chicken, mouse, human), other syntenic genes still map closely together (D, left side), which suggests that *Emx3* had been lost from that syntenic region. *Emx* genes are highlighted in *red*. Orthologs with widely conserved synteny are named and represented by bold lines on chromosomes. Orthologs are drawn on the same horizontal level except where dashed lines indicate inversions. Distances between genes are not drawn to scale. Numbers show the approximate locations of the chromosome segments in megabases. Thin lines or gaps on a chromosome indicate single or multiple genes between those with widely conserved synteny. Species abbreviations: Cmi, elephant shark; Dre, zebrafish; Gac, stickleback; Gga, chicken; Hsa, human; Lja, lamprey; Mdo, opossum; Mmu, mouse; Sca, dogfish; Tni, *tetraodon*; Tru, *fugu*; Xla, *Xenopus laevis*; Xtr, *Xenopus tropicalis*. Full species names and accession numbers are given in the Experimental Procedures section.

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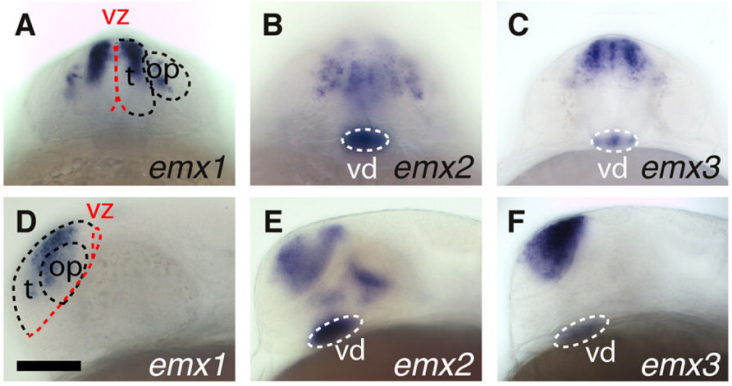

Fig. 2.

emx1, *emx2*, and *emx3* mRNA is expressed in overlapping domains in the dorsal telencephalon. **A–C:** *emx1* (A) is expressed exclusively in the telencephalon (t) mantle, away from the ventricular zone, whereas *emx2* (B) and *emx3* (C) are expressed in both the ventricular zone (vz, *red* dashed line) and mantle. vd (*white* dashed lines) indicates the ventral diencephalic expression domain of *emx2* and *emx3*. *emx1*, *emx2*, and *emx3* are also expressed in few cells of the olfactory placode (op in A,D). **D–F:** Side views are included for orientation. The *red* dashed line in D outlines the fissure separating telencephalon (t) and diencephalon; *black* dashed lines outline the telencephalon and olfactory placode in A,D. A–C: Frontal views. D–F: Side views. Scale bar = 50 μm.

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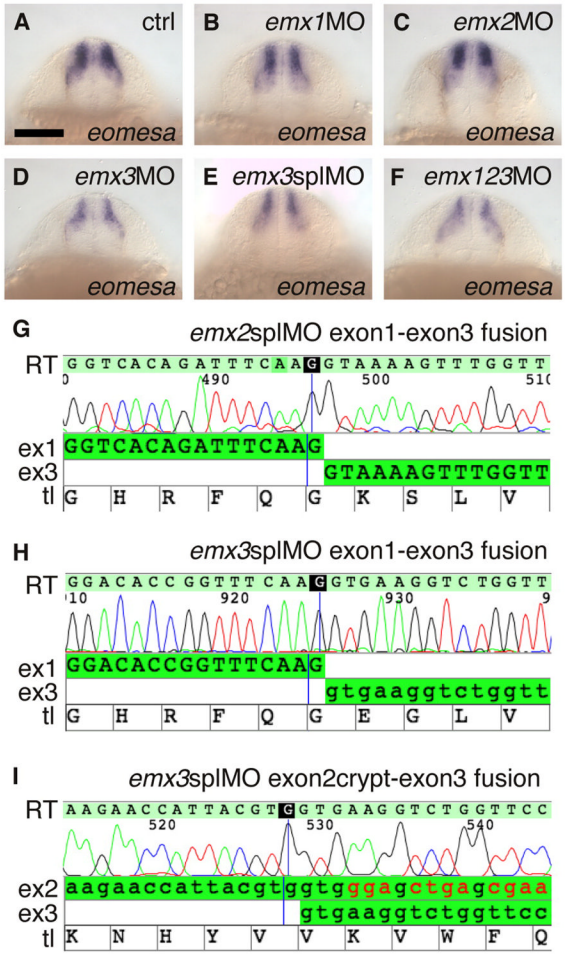

Fig. 3.

Only *emx3* and *emx1* regulate expression of *eomesa*. **A–E:** Injection of *emx3* morpholino antisense oligonucleotides (MO; D,E), to a lesser extent *emx1*MO (B), but not *emx2*MO (C), leads to reduced expression of *eomesa* compared with control embryos (A). **F:** The *emx3* morpholino phenotype is not enhanced when *emx1*MO and *emx2*MO are coinjected. *emx3* splice morpholinos (E) give rise to the same phenotype as translation blocking morpholinos (D), and lead to aberrantly spliced mRNA (H,I), which together confirms specificity of *emx3* morpholino phenotypes. **G–I:** Reverse transcriptase-polymerase chain reaction (RT-PCR) of RNA from embryos injected with *emx2* and *emx3* splice morpholinos that target exon 2 leads to aberrantly spliced mRNAs. Exon1 is fused to exon3 of *emx2* (G) and *emx3* (H), respectively. Alternatively, the third exon of *emx3* is spliced to a cryptic splice site within exon 2 that leads to an in-frame deletion of parts of helix 2 of the homeodomain (I). A–F: Frontal views of 24 hr embryos, dorsal to the top. Scale bar = 100 μm.

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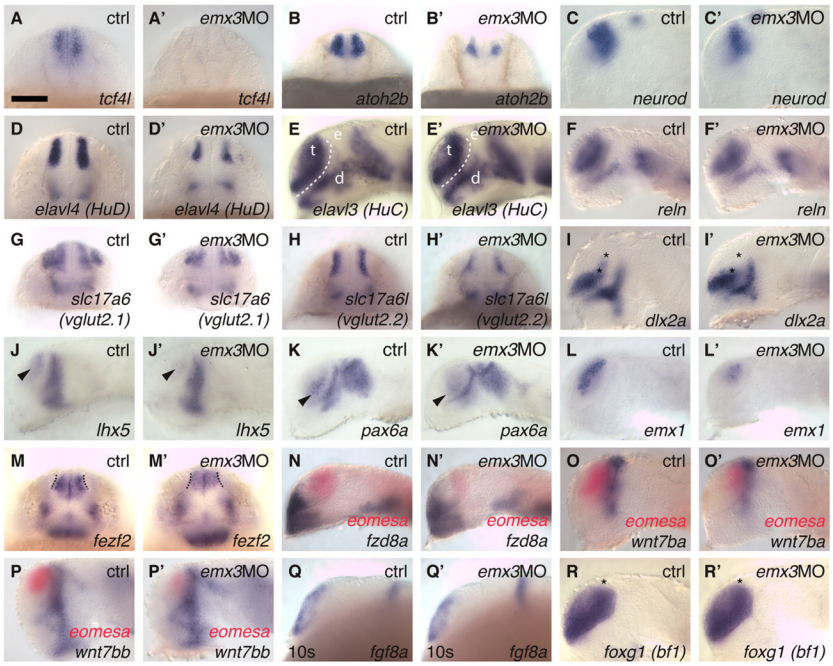

Fig. 4.

A–R: *emx3* morpholino injection specifically reduces expression of dorsal telencephalic marker genes. A–D′, G–N′: Expression of *tcf4l* (A–A′) in the dorsal telencephalon is lost, and expression domains of *atoh2b* (B–B′), *neurod* (C–C′), *elavl4* (D–D′), *slc17a6* (G–G′) *slc17a6l* (H–H′), *dlx2a* (I–I′, only the domain between asterisks), *lhx5* (J–J′), *pax6a* (K–K′), *emx1* (L–L′), are reduced in *emx3* morpholino antisense oligonucleotides (MO) injected embryos compared with control embryos. More broadly expressed neuronal differentiation markers *elavl3* (E–E′) and *reln* (F–F′) show little or no apparent difference between controls and *emx3*MO injected embryos. *fezf2* (M–M′) expression is reduced only in the more intense expression domain adjacent to the olfactory placodes (dotted lines), but unchanged in the ventricular zone. Ventral telencephalic expression domains (*dlx2a*, I–I′, *fzd8a*, N–N′; *fgf8a*, Q–Q′; *foxg1*, R–R′) are unaffected by *emx3*MO injections, as are diencephalic expression domains (C–K′, M–Q′). Shape and size of the telencephalon appears unaltered in *emx3*MO embryos compared with control embryos, as indicated for example by *elavl3* (E–E′; t, telencephalon, outlined by white dotted lines; e, epiphysis; d, diencephalon), and *foxg1* (R–R′) expression. Commonly used previous gene names of recently renamed genes are given in parentheses. A–B′, D–D′, G–H′, M–M′: Frontal views, dorsal to the top. C–C′, E–F′, I–L′, N–R′: Side views, dorsal to the top, rostral to the left; eyes removed except in Q–Q′. A–P′, R–R′: At 24 hr, (Q–Q′) 10-somite stage (14 hr). Scale bar = 100 μm.

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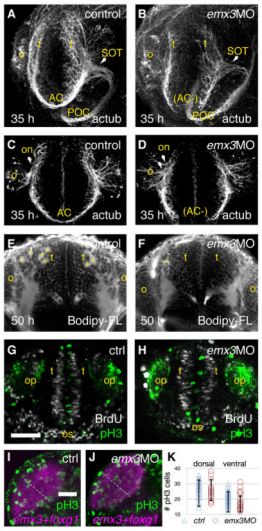

Fig. 5.

Embryos injected with *emx3* morpholino antisense oligonucleotides (MO) form abnormal axon tracts, lack olfactory glomeruli, and have normal cell division patterns. **A–D:** Acetylated tubulin labeling of axons; 35 hr; *emx3* knockdown embryos lack anterior commissure (AC in B,D). The supraoptic tract (SOT) is present but not fasciculated (arrows in A,B). Similarly, the olfactory nerve (on) that extends from the developing olfactory organ (o) to the dorsal telencephalon (t) is defasciculated in *emx3* knockdown embryos (arrows in C,D). **E,F:** *emx3*MO injected embryos have fewer presumptive olfactory glomeruli (*yellow* asterisks), visualized by Bodipyceramide staining. As in (G,H), the characteristic morphology of the ventricular zone appears broader in *emx3*MO embryos. **G,H:** Five minutes of 5-bromo-2-deoxyuridine (BrdU, *white*) incubation labels cells in S-phase. In control- and *emx3*MO-injected embryos at 24 hr, BrdU labeled cells are located in a 3–4 cell-wide region close to the ventricular zone. Mitotic cells, labeled by anti-phosphorylated histone H3 (pH3, *green*), are located on the ventricular side of the telencephalon in both *tp53*MO and *emx3*MO injected embryos. **I–K:** The number (K) and dorsoventral distribution (I–K) of pH3 positive cells in the medial telencephalic ventricular zone is the same in control (I,K) and *emx3*MO injected embryos (J,K). I,J: Projections of the side view confocal stack used for cell counts in K; the dotted line indicates the border between dorsal and ventral telencephalon, error bars denote SEM. Anterior is to the left, dorsal to the top. Confocal projections of oblique (A,B) straight (C–H) frontal views, dorsal to the top. os, optic stalk; POC, postoptic commissure. Scale bars = 50 μm.

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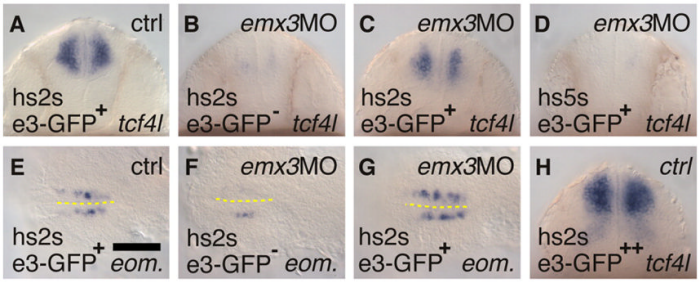

Fig. 6.

A–H: Heat shock-induced Emx3 protein partially rescues *emx3* morpholino antisense oligonucleotides (MO) induced knockdown, whereas excessive Emx3 dorsalizes the telencephalon. **A–C,E–G:** Low levels of Emx3-myc-GFP expression (e3-GFP^{+}), induced by heat shock at the 1- or 2-somite stages (10.5 hr, hs2s), 20–30 min after the onset of endogenous *emx3* mRNA expression at bud stage, rescues the *emx3*MO-induced down regulation of *tcf4l* (A–C) and *eomesa* (*eom.*, E–G). Later heat shock, between the 5- and 7-somite stages (11.5 hr, hs5s), does not rescue the phenotype (B,D). Higher levels of GFP expression after heat shock (e3-GFP^{++}), indicating higher levels of Emx3 activity, lead to dorsalized telencephalon as assessed by ventrally expanded expression of *tcf4l* (A,H). A–D,H: Frontal views, dorsal to the top, 25 hr; (E–G) Dorsal view, rostral to the left, 12-somite stage (15 hr). Scale bar = 100 μm.

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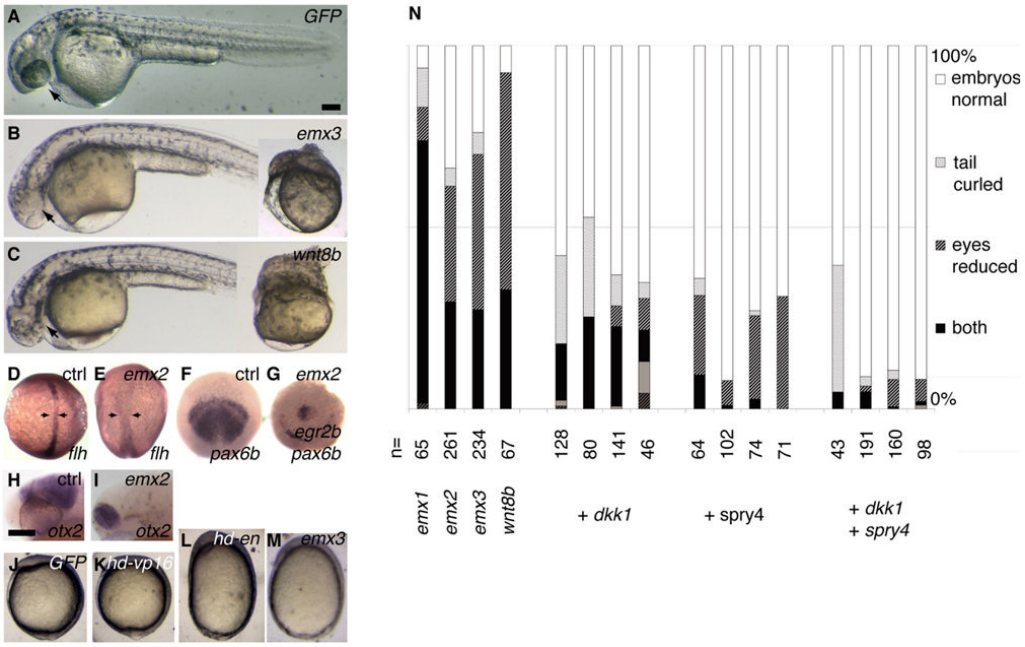

Fig. 7.

A–N: Overexpression of *emx1*, *emx2*, or *emx3* mRNA, as well as *emx3*-homeodomainen mRNA, dorsalizes and posteriorizes embryos. A–I,J,M: Injection of emx2 mRNA (E,G,I), emx3 mRNA (B,M), and *wnt8b* mRNA (C) produces embryos with posteriorized central nervous systems (loss of eyes in B,C,I loss of *pax6b* expression and anterior shift of *egr2b* expression in G; loss of otx2 expression except for an abnormally anterior expression domain in I), and dorsalization phenotypes (curly tail in B,C, insets, broadened flh-expressing notochord in E, abnormally egg-shaped embryos in E,M). J–M: Dorsalization effects are mimicked by *emx3hd-en* (L), but not *emx3hd-VP16* (K) fusion proteins, indicating that *emx3* might act as a repressor. N: Quantification of phenotypes caused by *emx* and *wnt8b* mRNA injection and their rescue by coinjection of the Wnt/beta-catenin pathway inhibitor *dkk1* and Fgf pathway inhibitor *spry4*, suggesting that excessive *emx* mRNA in the early embryo potentiates the effects of these signaling pathways. A–C,H–M: Side views, dorsal to the top, rostral to the left; (D,E) Dorsal views, animal pole to the top; (F,G) Animal pole views, dorsal to the bottom. (A–C) 35 hr; (H,I) 30 hr; (D–G, J–M) bud stage. Scale bars = 100 μm.

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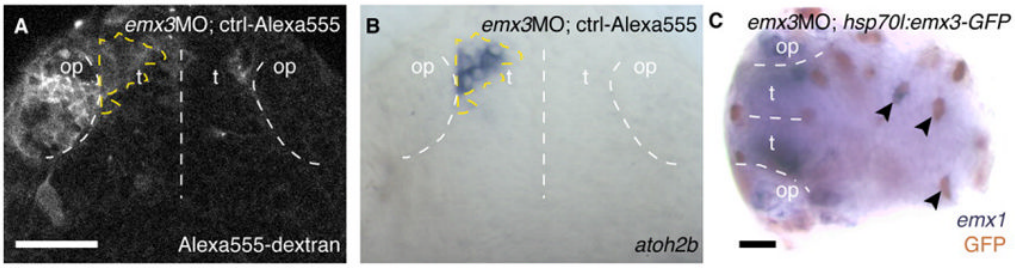

Fig. 8.

emx3 is required cell-autonomously for *atoh2b* and *emx1* expression. **A,B:** *tp53* morpholino antisense oligonucleotides (MO) and Alexa555-dextran-injected donor cells (A,B, *yellow* dotted line), but not *emx3*MO plus *tp53*MO injected host cells, express *atoh2b* (B) in chimeric embryos. *tp53*MO cells were co-injected with Alexa555-dextran and transplanted into *emx3*MO injected host embryos. **C:** Ectopic expression of *emx3:GFP* fusion (brown), under the control of *hsp70l* promoter, induces ectopic expression of *emx1* (blue) in a cell-autonomous manner. White dotted lines outline olfactory placode borders and telencephalon midline. op, olfactory placode; t, telencephalon. (A,B) Frontal views, dorsal to the top; (C) dorsal view, rostral to the left; (A,B) 24 hr, (C) 20 hr. Scale bar = 50 μm.