MID1 and MID2 are required for Xenopus neural tube closure through the regulation of microtubule organization

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SUMMARY
Closure of the neural tube requires both the change and maintenance of cell shape. The change occurs mainly through two coordinated morphogenetic events: cell elongation and apical constriction. How cytoskeletal elements, including microtubules, are regulated in this process in vivo is largely unknown. Here, we show that neural tube closure in Xenopus depends on orthologs of two proteins: MID1, which is responsible for Opitz G/BBB syndrome in humans, and its paralog MID2. Depletion of the Xenopus MIDs (xMIDs) by morpholino-mediated knockdown disrupted epithelial morphology in the neural plate, leading to neural tube defects. In the xMID-depleted neural plate, the normal epithelial organization was perturbed without affecting neural fate. Furthermore, the xMID knockdown destabilized and caused the disorganization of microtubules, which are normally apicobasally polarized, accounting for the abnormal phenotypes. We also found that the xMIDs and their interacting protein Mig12 were coordinately required for microtubule stabilization during remodeling of the neural plate. Finally, we showed that the xMIDs are required for the formation of multiple epithelial organs. We propose that similar MID-governed mechanisms underlie the normal morphogenesis of epithelial tissues and organs, including the tissues affected in patients with Opitz G/BBB syndrome.

KEY WORDS: Neural tube closure, Microtubule, MID1, MID2, Opitz syndrome, Epithelial remodeling, Xenopus

INTRODUCTION
In vertebrates, the neural tube is the primary luminal structure of early development. It is the anlage of the central nervous system and forms from a flat neuroepithelial sheet called the neural plate. The lateral edges of the neural plate form ridges, called neural folds, along the dorsal surface, parallel to the anterior-posterior axis. The neural folds continue to rise and eventually meet at the dorsal midline, where they fuse to form the luminal structure of the neural tube (Colas and Schoenwolf, 2001; Copp et al., 2003; Davidson and Keller, 1999). Failure of neural tube closure causes congenital malformations, collectively called neural tube defects (NTDs), including anencephaly and spina bifida (Colas and Schoenwolf, 2001; Copp et al., 2003).

During neural tube closure, the neuroepithelial cells undergo dynamic changes in shape, including apicobasal elongation and apical constriction, which cause the tissue to bend and form the neural tube (Colas and Schoenwolf, 2001; Davidson and Keller, 1999). The apicobasal elongation changes the cuboidal neuroepithelial cells into columnar cells (Burnside, 1973). Apical constriction minimizes the apical surface of selected cells in the neural plate (located at hinge points), causing them to adopt wedge-like rather than columnar shapes (Schoenwolf and Franks, 1984). To achieve the complex morphological changes required for tube formation, these cellular changes must be tightly controlled in time and space.

It is widely accepted that regulation of the neuroepithelial cytoskeleton is fundamental to cellular morphogenesis during neural tube closure (Colas and Schoenwolf, 2001; Copp et al., 2003; Pilot and Lecuit, 2005; Quintin et al., 2008). In particular, regulation of the actin cytoskeleton has been extensively studied, and analyses in mice, chick and Xenopus show that actin-binding proteins and their regulators, including Shroom3, MARCKS, Rap1, ROCKs, p190 RhoGAP and RhoA, positively regulate apical constriction via myosin activity (Copp et al., 2003; Haigo et al., 2003; Hildebrand, 2005; Kinoshita et al., 2008; Nishimura and Takeichi, 2008). In addition, cell adhesion molecules such as N-cadherin and Nectin contribute to apical constriction by regulating cortical actin assembly (Morita et al., 2010; Nandasa et al., 2009).

By contrast, the roles and regulatory mechanisms of microtubules in neural tube closure have been elusive. During cell elongation, microtubules polymerize and assemble along the apicobasal axis (Burnside, 1973; Handel and Roth, 1971; Karfunkel, 1971). In chick and Xenopus, microtubule polymerization inhibitors induce aberrant cell morphologies and defects in neural tube closure (Handel and Roth, 1971; Karfunkel, 1971). In addition, non-centrosomal γ-tubulin, indirectly recruited to the apical side by Shroom3, participates in the assembly of microtubule arrays and apicobasal cell elongation (Lee et al., 2007). Thus, microtubules appear to be important in the cellular morphogenesis required for neural tube closure.

Here, we show that the Xenopus orthologs of human MID1 (also known as FXY, RNF59, TRIM18) and of MID2, an MID1 paralog (also known as FXY2, RNF60, TRIM1) are crucial for epithelial remodeling in neural tube closure. In humans, MID1 is responsible for X-linked Opitz G/BBB syndrome (OS), listed as OMIM 30000 (Buchner et al., 1999; Quaderi et al., 1997; Robin et al., 1995). OS is characterized by midline malformations, including hypertelorism, hypospadias, cleft lip/palate, ...
laryngotracheoesophageal abnormalities, imperforate anus, cardiac defects and brain abnormalities (Fontanella et al., 2008; So et al., 2005).

**MID1 and MID2 encode conserved proteins associated with microtubules belonging to the RBCC/TRIM (N-terminal RING finger-B box-coiled coil/tripartite motif) superfamily (Buchner et al., 1999; Cainarca et al., 1999; Schweiger et al., 1999; Short and Cox, 2006). MID1 and MID2 are known to be expressed during development in human, mouse and chick (Buchner et al., 1999; Dal Zotto et al., 1998; Granata et al., 2005; Pinson et al., 2004; Quaderi et al., 1997; Richman et al., 2002). However, although biochemical and in vitro cell biological studies have yielded some information, the physiological and developmental functions of the MID proteins are still unclear, as is the pathological role of the MID1 mutant in OS. We report here that *Xenopus* MID1 and MID2 (XMID1 and XMID2) are essential for neural tube closure through their stabilization of microtubules, which is required for cell elongation and apical constriction. We propose that microtubule regulation by the MIDs is crucial for a variety of epithelial remodeling processes during the development of many vertebrate species.

**MATERIALS AND METHODS**

**Cloning of *Xenopus MID1* and *MID2***

*Xenopus laevis* MID1 was identified as a cDNA clone, XL082d10, in our EST database (XDB3, http://xenopus.nibb.ac.jp). Since this clone contains a 103 bp internal non-coding sequence, we isolated the entire coding region from neurula cDNA by PCR using UTR sequence-specific primers (5'-ggattgGACAGGGCTGTATTCTC-3' and 5'-TGTCATTGCAATGGATTCCCAATGGC-3'), and cloned it into pCS2+. Similarly, a partial cDNA of *Xenopus laevis* MID2 was obtained by PCR from neurula cDNA using primers based on the genomic sequence of *Xenopus tropicalis* (5'-GAATGGGAGCAGCCCTGCTCATTCT-3' and 5'-ACCTCAGGAATTTCTTCTGGAATTTTGCTTAC-3'), and cloned into pCS2+. Phylogenetic analysis was performed using MEGA4 software (Tamura et al., 2007). GenBank accession numbers are GU362929 (XMID1) and GU362930 (XMID2).

**Morpholinos, plasmids and mRNA preparation**

Antisense morpholino oligonucleotides (Mo) were obtained from Gene Tools. The Mo sequences were as follows: xMID-Mo, 5'-CAGTGCATTGCAATGGATTCCCAATGGC-3'; standard control-Mo, 5'-GGATGAGAACACTTATGCACCAC-3'. Ten plasminos were used for each experiment. The following primers were used: xMID1, 5'-GGTTGCTCTCTCTGTGATATAATGGTCTCTCTCTG-3'; MID2, 5'-GTGATGAAGGTAAGAAACATTGCGTC-3' and 5'-ACCTCAGAACATTTCTCCTGCTG-3'; NCAM, 5'-GGCTGTAGAATTACAATGCTG-3'; Sox2, 5'-GAGGATGAGAACACTTATGCACCAC-3'; and 5'-GGACATCGTGTAAGTAGGCGGA-3'; Epidermal keratin 1, 5'-CCTGTTGAAGAATTAACCTGA-3'; and 5'-GAACCTTCCATCAATCCCACAAC-3'; ODC, 5'-CAGTCATGTTGTTGGA-3' and 5'-CACATCGGAAATCTCACCAC-3'. The following plasmids were used for probe synthesis: XMID1 and XMID2 (constructed for this study); Sox2 (XL039b04, XDB3); NCAM (Kintner and Melton, 1987); N-cadherin (XL289n05, XDB3); Epidermal keratin 1 (XL056e18, XDB3); Shh (Yakushiji et al., 2007); Ptc2 (Yakushiji et al., 2007); Glia (Takabatake et al., 2000); Glia3 (Takabatake et al., 2000); HNF3b (Foxa2a) (XL01611, XDB3); Pintallavis (Foxa4a) (XL047n03, XDB3); N-tubulin (Takabatake et al., 2002); Shroom3 (Haigo et al., 2003); and Pax3 (XL014p10, XDB3).

**Western blotting and immunoprecipitation**

For western blotting to test the specificity of xMID-Mo, 20 embryos at stage 14 were lysed in 400 μl lysis buffer [30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 50 mM NaF, protease inhibitors]. For immunoprecipitation of EGFP-tubulin, 30 embryos at the late neurula stage were lysed in 600 μl lysis buffer. Immunoprecipitation was performed as described (Okawara et al., 2003). Antibodies to GFP (598, MBL) and acetylated tubulin (T6793, Sigma) were used.

**Immunohistochemistry**

Embryos were fixed in Dent’s Fixative (for staining with antibodies) or MEMFA. Published procedures were used to prepare (Fagotto and Gumbiner, 1994) and stain (Suzuki et al., 2007) fish gelatin cryosections, or thick sections (Becker and Gard, 2006), with minor modifications. Antibodies to the following proteins were used: Sox2 (AB5603, Chemicon); phospho-histone H3 (06-570, Upstate); actin (constructed for this study); and 0.5 pg activin mRNA was injected into the animal pole of two-cell embryos. The animal cap was dissected at stage 9 and cultured in Steinberg’s Solution until the sibling embryos reached stage 17.

**Embryo manipulation and microinjection**

Capped mRNAs or Mo were injected into the appropriate region of two- or four-cell embryos. The injected embryos were cultured in 3% Ficoll/0.1X Steinberg’s Solution to stage 9, then washed and cultured in 0.3X Marc’s Modified Ringer’s (MMR) until the appropriate stage (Nieuwkoop and Faber, 1967). Morphogenetic defects in the morphants were analyzed at stage 16-17 unless otherwise stated. In animal cap elongation assays, 0.5 pg activin mRNA was injected into the animal pole of two-cell embryos. The animal cap was dissected at stage 9 and cultured in Steinberg’s Solution until the sibling embryos reached stage 17.

**RT-PCR and in situ hybridization**

RT-PCR and in situ hybridization were performed as described (Goda et al., 2009). For RT-PCR with dissected tissues, the neural plate and ventral epidermis at stage 14 were separated from the underlying mesoderm in Danilich’s For Amy Medium (DFA) (Sater et al., 1993). Ten plasminos were used for each experiment. The following primers were used: xMID1, 5'-GGTTGCTCTCTCTGTGATATAATGGTCTCTCTCTG-3'; MID2, 5'-GTGATGAAGGTAAGAAACATTGCGTC-3' and 5'-ACCTCAGAACATTTCTCCTGCTG-3'; NCAM, 5'-GGCTGTAGAATTACAATGCTG-3'; Sox2, 5'-GAGGATGAGAACACTTATGCACCAC-3'; and 5'-GGACATCGTGTAAGTAGGCGGA-3'; Epidermal keratin 1, 5'-CCTGTTGAAGAATTAACCTGA-3'; and 5'-GAACCTTCCATCAATCCCACAAC-3'; ODC, 5'-CAGTCATGTTGTTGGA-3' and 5'-CACATCGGAAATCTCACCAC-3'. The following plasmids were used for probe synthesis: XMID1 and XMID2 (constructed for this study); Sox2 (XL039b04, XDB3); NCAM (Kintner and Melton, 1987); N-cadherin (XL289n05, XDB3); Epidermal keratin 1 (XL056e18, XDB3); Shh (Yakushiji et al., 2007); Ptc2 (Yakushiji et al., 2007); Glia (Takabatake et al., 2000); Glia3 (Takabatake et al., 2000); HNF3b (Foxa2a) (XL01611, XDB3); Pintallavis (Foxa4a) (XL047n03, XDB3); N-tubulin (Takabatake et al., 2002); Shroom3 (Haigo et al., 2003); and Pax3 (XL014p10, XDB3).

**Imaging and image analysis**

For rescue experiments, we co-injected 0.25% Rhodamine-Dextran (D1817, MBL) and acetylated tubulin (TT6793, Sigma) were used.
Fig. 1. Expression patterns of *xMID1* and *xMID2* through early embryogenesis. (A,B) RT-PCR analysis. (A) *xMID1* and *xMID2* mRNAs are expressed maternally and zygotically. *Ornithine decarboxylase* (*ODC*) was used as an internal control. The number above each lane is the embryonic stage. –RT, control experiment without reverse transcriptase. (B) Ectodermal expression of *xMIDs* in the early neurula (stage 14) *Xenopus* embryo. In the neural plate, *xMID1* and *xMID2* were expressed at similar levels to in ventral epidermis (Epidermis) and whole embryo (W.E.). *NCAM* and *Sox2* are pan-neural markers. *Epidermal keratin 1* (*Epi. keratin*) is an epidermal marker. (C) Expression pattern of *xMID1* in embryos at the stages indicated (lower right in each panel). (D) Dorsal view, dorsal to the top. (E) Dorsal view, anterior to the top. (F) Anterior view, dorsal to the top. (G) Dorsal view, labeled with sense probe (S), anterior to the top. (H,I) Lateral view, anterior to the left, dorsal to the top. (J,K) *xMID2* expression. Lateral view, anterior to the left, dorsal to the top. (K) Sense probe (S): cg, cement gland; hb, hindbrain; ht, heart tube; mb, midbrain; nt, neural tube; op, optic vesicle; ot, otic vesicle; pg, pineal gland; pn, pronephros; pp, pharyngeal pouch; pr, proctodeum; so, somite.

between the neural folds marked by pigmented margins in the anterior spinal cord anlage was measured. To quantify the cellular characteristics, images of stained sections were obtained with a Zeiss LSM510 META confocal microscope equipped with a 63×, NA 1.4, oil-immersion or a 40×, NA 1.2, water-immersion objective lens. The cell height, apical width, basal width, perimeter of the apical side, and pixel intensities were all determined using ImageJ (NIH) software (see Fig. S4E in the supplementary material). To quantify the cell morphologies, we selected cells with easily detected tracer fluorescence and a visible nucleus. The apical width and perimeter were defined as the distance and outline length between apical cell–cell junctions, respectively. The cell height was defined as the maximal length along the axis perpendicular to the apical width. Similarly, the basal width was defined as the distance between the basal cell-cell junction with the neighboring cell and the line perpendicular to the apical width in contact with the opposite basal junction. Cell width was defined as the larger of the apical and basal widths (see Fig. S4E in the supplementary material).

To quantify molecular markers, labeled areas in the apical cell junctions (ZO-1, C-cadherin, β-catenin) or the basal lamina (laminin) were manually selected to exclude background staining. For apical cell junctions, we selected the region with higher fluorescence intensity than detected more basally, and excluded almost all of the cell membrane with baseline-level staining and the cytoplasm, because we could not clearly distinguish between low-level specific signals and non-specific background labeling. Then, the total pixel intensity in each selected area was measured. In the case of laminin, we drew a thin (one-pixel-wide) line along the basal end of the cell, at the level of the basal lamina, where the laminin fluorescence was brightest, and measured the fluorescence intensity on the line. Since the basal width varied greatly among cells, we normalized the data by dividing the total intensity by the length of the line. Data were analyzed by Student’s *t*-test, and are presented as the mean ± s.e.m.

**RESULTS**

**Identification of the *Xenopus* homologs of *MID1* and *MID2***

To identify candidate molecules for regulating neural tube closure, we focused on MIDs that were implicated in epithelial morphogenesis through microtubule regulation. We isolated two cDNAs that encode proteins of 668 and 687 amino acids and exhibit 92% identity to human *MID1* and 83% to human *MID2*, respectively (see Fig. S1A in the supplementary material). A phylogenetic analysis confirmed that the two genes were closest to human *MID1* and *MID2* (see Fig. S1B in supplementary material) and we therefore named them *xMID1* and *xMID2*.

By reverse transcription PCR (RT-PCR), we found that both *xMIDs* were expressed throughout embryogenesis (Fig. 1A). In addition, both genes were expressed in the neural plate of the early neurula (Fig. 1B). Next, we analyzed the spatial expression of the *xMIDs* in detail by in situ hybridization. Before neurulation, neither gene was detectable (Fig. 1C; data not shown). During early to mid-neurulation, *xMID1* was upregulated uniformly in the embryo (Fig. 1D), and by late neurulation its transcripts were detected in the epithelial organs, including the neural tube, optic and otic vesicles, cement gland and newly epithelialized somites (Fig. 1E,F). At the tailbud stages, additional tissues expressed *xMID1*, including the midbrain, hindbrain, pronephros, pharyngeal pouch, heart tube and scattered epidermal cells (Fig. 1H,I). By contrast, expression of *xMID2* was undetectable at neurula stages (data not shown), whereas weak expression was observed in the pineal gland, otic vesicle and heart tube at the tailbud stages (Fig. 1J).
Knockdown of xMIDs causes neural tube defects

To deplete the endogenous xMID proteins and elucidate their in vivo role, we designed a specific antisense morpholino oligonucleotide (xMID-Mo) that efficiently blocked the translation of not only xMID1-Venus, but also xMID2-Venus, owing to the similarity of the Mo recognition site (Fig. 2A,B). The xMID-Mo did not reduce the protein level of a complementary sequence of the xMID-Mo compared with xMID1 and xMID2. The ATG codons for the first methionine are underlined. xMID-Mo caused severe defects in which the neural tube remained open even at the late neurula stage (Fig. 2E). In addition, xMID morphant cells were consistently found as a dissociated clump of cells at the anterior neural plate [96% (n=73 embryos) compared with 0.02% (n=95 embryos) in control morphants] (Fig. 2E). Coinjection of the xMID-Mo with the mRNAs of rescue constructs with silent mutations in the Mo recognition site of xMID-Mo (Fig. 2F) did not block translation of the mRNA for Venus (Vns, 100 pg) or the mRNA for xMID-Venus with six silent mutations in the Mo recognition site (mut-xMID1-Vns, 250 pg). (C,D) Dorsal views of the unilaterally injected morphants; anterior is to the top. Control-Mo (C) or xMID-Mo (D) was injected into the right dorsal blastomere at the four-cell stage. Dashed lines indicate the boundaries between the neural and non-neural ectoderm. The black bracket marked by an asterisk indicates the distance between the neural folds in a rescue experiment. (E) Dorsal view of bilaterally injected xMID morphants at stage 20; anterior is to the top. (F,G) Transverse sections through the neural plate of Xenopus embryos with unilateral injection of control-Mo (F) or xMID-Mo (G). Dashed lines indicate the outlines of neural tissues, and brackets show the distance between the neural folds in a rescue experiment. (H) Average distance between the neural folds of unilaterally injected xMID morphants were dose-dependently reduced by the Mo-insensitive xMID1 (50, 100, 200, 500 pg), xMID2 (50, 100, 200, 500 pg), or xMID1+2 (25, 50, 100, 250 pg each) mRNAs. The number of embryos examined is indicated above each bar.

Knockdown of xMIDs does not affect gastrulation movements, cell viability, neural development or primary ciliogenesis

To investigate whether the effects of xMID-Mo were specific to epithelial remodeling, we examined gastrulation movement, cell viability and neural specification and patterning. xMID-Mo did not affect gastrulation movement, activin-induced animal cap elongation or cell proliferation and viability as revealed by staining for phospho-histone H3 and the apoptosis marker active caspase 3 (see Fig. S2 in the supplementary material). From these results, we concluded that the loss of xMID function did not affect gastrulation, cell viability or neural differentiation. Since xMID1 and xMID2 repress Shh in Hensen’s node in chicken (Granata and Quaderi, 2003; Granata et al., 2005), and a deficiency in Shh activity inhibits neural plate bending (Ybot-Gonzalez et al., 2002), we examined neural specification, dorsoventral patterning and the Shh pathway in the xMID morphants. We detected no apparent change in the markers tested, except that the delayed neural tube closure resulted in expression domains that were wider than normal at these stages (see Fig. S2F and Fig. S3 in the supplementary material). From these results, we concluded that the loss of xMID function did not affect gastrulation, cell viability or neural specification and patterning.

Recent studies have shown that mutations in ciliary genes that result in agenesis of the primary cilium, a microtubule-based organelle, cause NTDs, indicating some linkage between primary cilium formation and neural tube closure (Bisgrove and Yost, 2006). Hence, we analyzed the genesis of the primary cilia in the neural tube. Knockdown of the xMIDs did not obviously affect the length...
or number of primary cilia (see Fig. S2F in the supplementary material). Therefore, the NTDs of the xMID morphants are not attributable to a defect in primary cilium formation.

**Knockdown of xMIDs induces aberrant cell morphology in the neural plate**

Next, we analyzed the epithelial cell morphology in the xMID morphants by phalloidin staining. On the control side, the neuroepithelial cells showed normal apicobasal elongation and apical constriction (Fig. 3A,A', blue outlines). In striking contrast, the xMID morphant cells did not elongate, but remained rounded, and their apical constriction was perturbed (Fig. 3A-A', pink outlines; see also Fig. S4A-D in the supplementary material). Consistent with this, the cortical actin, the assembly of which is a prominent feature of apical constriction, was clearly attenuated (Fig. 3A, arrowheads).

To quantify the morphological defects in the xMID morphants (see Fig. S4E in the supplementary material), we analyzed the neuroepithelial cells in the superficial and deep layers separately (Schroeder, 1970). In the xMID-depleted superficial layer, the cell height and apical width were significantly decreased and increased, respectively, compared with the control (Fig. 3B-D). Consequently, the ratios of cell height to width and apical width to basal width were altered (Fig. 3E,F). We observed similar defects in the deep layer, except that the basal width of the deep morphant cells was significantly increased (see Fig. S4F-J in the supplementary material). Therefore, the NTDs of the xMID morphants are not attributable to a defect in primary cilium formation, and the exogenous xMIDs rescued the cell morphology from a rounded to a columnar shape. These data strongly suggest that the NTDs of the xMID morphants were due to defects in cellular morphogenesis in the neural plate.

**Defects in cell-cell and cell-extracellular matrix contacts in the neural plate**

To dissect the cellular phenotype of the xMID morphants, we examined the localization of proteins involved in cell-cell and cell-extracellular matrix (ECM) contacts. In the control cells, ZO-1 (also known as TJP1), a tight junction marker (Itoh et al., 1993), and C-cadherin and β-catenin, the major components of the cadherin complex (Briicher and Gumbiner, 1994; Levine et al., 1994), were concentrated at the apical junction (Fig. 4A,C,E). By contrast, at the apical junction in the xMID morphant cells, the ZO-1 signal was obscure (Fig. 4B,I) and the levels of C-cadherin and β-catenin were severely reduced (Fig. 4D,F,J,K), although the transcription and translation of these molecules were unaffected (data not shown). We also examined laminin, a major component of the basal lamina (Miner et al., 1998), and found that its localization in the basal lamina was attenuated in the xMID morphants (Fig. 4G,H,H',L). Thus, the knockdown of xMIDs resulted in the aberrant organization of cell-adhesive machineries and the polarized distribution of the ECM.

**Defective microtubule organization and stabilization in xMID morphants**

We next analyzed the subcellular localization of EGFP-tagged xMID1. Interestingly, EGFP-xMID1 colocalized with bundles of non-centrosomal microtubules stained with anti-α-tubulin antibody (Fig. 5A), which are readily observed in apicobasally elongated epithelial cells (Bacallao et al., 1989; Bartolini and Gundersen, 2006; Lee et al., 2007). In the control columnar epithelial cells, the apicobasal arrays of microtubules were also readily apparent (100%, n=11 cells, three embryos) (Fig. 5B). By contrast, in the xMID morphant cells, the arrays of microtubules...
were not polarized, and the cells were more rounded (85%, n = 13 cells, four embryos) (Fig. 5D). To assess the stability of the polymerized microtubules, we analyzed their acetylation status (Creppe et al., 2009; Verhey and Gaertig, 2007). In the control cells, filamentous and continuous staining was detected, particularly in the apical region (86%, n = 7 cells, two embryos) (Fig. 5C). By contrast, in the rounded xMID morphant cells the acetylated α-tubulin staining was punctate (92%, n = 13 cells, four embryos) (Fig. 5E). Furthermore, the acetylation of the overexpressed EGFP-tubulin was markedly decreased in the xMID morphants (40 ± 4.5%, P < 0.01, n = 3) (Fig. 5F,G). Thus, the microtubules of the xMID morphant cells were disorganized and destabilized.

We next examined the relationship between the microtubule destabilization and the NTDs in the morphant embryos by co-injecting the xMID-Mo with the mRNA for an unrelated microtubule-stabilizing factor, tau (also known as MAPT), which is a classical microtubule-associated protein (Kanai et al., 1992; Lu and Kosik, 2001). The forced expression of tau not only rescued the disrupted neural cell morphologies (see Fig. S5C,D,G-L in the supplementary material), but also partially rescued the NTDs of the xMID morphants (see Fig. S5A,B in the supplementary material). Furthermore, the apicobasal polarization of the microtubule arrays was restored in the tau-injected xMID morphant cells (see Fig. S5E,F in the supplementary material). These findings suggest that xMIDs are required for the stabilization of microtubules.

**xMIDs functionally interact with Mig12 in neural tube closure**

*Mig12* (also known as *G12-like* and *MID11P1*), which encodes a MID1-interacting molecule, is expressed in the ventral midline of the neural plate (Berti et al., 2004; Conway, 1995; Hayes et al., 2007). The Mo-mediated knockdown of Mig12 causes NTDs (Fig. 6A-C) (Hayes et al., 2007) and defects in epidermal ciliogenesis (Hayes et al., 2007). To investigate the functional interaction of xMIDs and Mig12, we performed individual injections or co-injections of xMID-Mo and Mig12-Mo. In morphants that received either Mo alone at a low dose, only a slight delay in neural tube closure was induced (Fig. 6D-F,H). By contrast, the co-injection of xMID-Mo and Mig12-Mo at the same low dose induced severe NTDs (Fig. 6G,H), suggesting that xMIDs and Mig12 interact functionally. We then performed rescue experiments of xMID morphants with *Mig12* and vice versa. The NTDs of the Mig12 morphants were rescued by *Mig12* mRNA in a dose-dependent manner (Fig. 6I). However, the NTDs in the xMID morphants were not rescued by *Mig12* mRNA (Fig. 6J), indicating that Mig12 requires the xMIDs to function in neural tube closure.

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**Fig. 4.** xMIDs regulate the localization of adhesive molecules. (A-H') Transverse sections through the neural plate of unilaterally control-Mo-injected (Cont.-Mo) (A,C,E,G) and xMID-Mo-injected (B,D,F,H) Xenopus embryos at stage 15.5, stained with antibodies against ZO-1 (A,B), C-cadherin (C-D), β-catenin (β-cat.) (E,F), or laminin (G,H). Flag-β-globin mRNA (250 pg) was co-injected as a tracer, and stained with an anti-Flag antibody (magenta). (A-F) Dashed lines indicate Flag-positive Mo-injected cells. (H) Arrowheads indicate the attenuation of laminin accumulation basal to the xMID-Mo-injected cells. (H') Higher-magnification view of the boxed area in H. Scale bars: 50 μm. (I-L) Quantification of marker intensities in the morphants at stage 15.5. For ZO-1 intensity: control-Mo, n = 27 sites (3 embryos); xMID-Mo, n = 44 sites (6 embryos). For C-cadherin: control-Mo, n = 16 sites (4 embryos); xMID-Mo, n = 20 sites (5 embryos). For β-catenin: control-Mo, n = 7 sites (3 embryos); xMID-Mo, n = 15 sites (4 embryos). For laminin: control-Mo, n = 19 sites (4 embryos); xMID-Mo, n = 19 sites (3 embryos). *P < 0.05, **P < 0.001. (M,N) Schematic illustrations showing the control (M) and xMID (N) morphants. Rectangles indicate the regions analyzed in this study. so, somite; nt, notochord.
Microtubule and xMIDs in neurulation

The neuroepithelial cells of the Mig12 morphants and xMID/Mig12 double morphants resembled those of the xMID morphants (Fig. 6K-M). Consistent with this, the assembly of apicobasally polarized microtubules was decreased in the double morphants (Fig. 6K-M, bottom). These results highlight the functional relationship between the xMIDs and Mig12 in regulating microtubule organization and cellular morphogenesis.

In vitro, when co-expressed with MID1, Mig12 colocalizes with microtubules and stabilizes them (Berti et al., 2004). To investigate the subcellular localization of Mig12, we expressed Mig12-GFP with xMID1 in animal caps and the neural plate (see Fig. S6 in the supplementary material). When expressed alone, Mig12 was distributed throughout the cells of the animal cap and neural plate (see Fig. S6A,C,D in the supplementary material). However, when co-expressed with xMID1, Mig12-GFP colocalized with the microtubules in the animal cap cells, although such colocalization was not evident in neuroepithelial cells (see Fig. S6B,E in the supplementary material). Thus, the functional interaction of the xMIDs and Mig12 appears to be highly dynamic and context-dependent, and in the neuroepithelial cells controlled physical and functional interactions allow highly organized microtubule remodeling.

xMIDs contribute to the development of other epithelial organs

To further investigate the role of xMIDs in epithelial morphogenesis, we performed targeted Mo injections into the presumptive head region of embryos and found that xMID-Mo caused developmental defects of the eye (data not shown). Characterization using a neural marker, Xen1, and a notochord marker, MZ15, revealed hypoplasia of the anterior central nervous system in the xMID morphants, although the notochord formed normally (Fig. 7A,B, insets; data not shown). We also analyzed neuroepithelial cell morphologies and laminin localization in the basal lamina at the tailbud stage. The normally multi-layered structures of the brain and optic vesicles (Fig. 7A,C) were disorganized, and the neuroepithelial cells had not elongated (Fig. 7B,D). Furthermore, neuroepithelial cells had dissociated from the apical surface of the epithelial sheet, which lacked actin filaments (Fig. 7L; data not shown). The neuroepithelial cells found in the ventricle were positive for active caspase 3 (Fig. 7K,L, dashed line), suggesting anoikis, a form of apoptosis caused by the loss of cell adhesion (Frisch and Screaton, 2001). Moreover, a continuous basal ECM failed to form, as indicated by the non-continuous and attenuated laminin staining (Fig. 7B,D, arrowheads). Thus, the NTDs that develop in xMID morphants ultimately cause the catastrophic collapse of the central nervous system.

Similar defects in cell morphogenesis were found in the cement gland (Fig. 7E,F), where xMID1 is strongly expressed (Fig. 1F,H,J). Furthermore, in the pronephros, the epithelial cells failed to adopt a columnar shape or exhibit apical actin assembly, and no tubular structure was formed (Fig. 7G,H). The area in which the pronephros normally forms was filled with disorganized cell aggregates (Fig. 7H). In the foregut, derivatives of which are affected in OS patients (Fontanella et al., 2008; So et al., 2005), continuous apical actin staining (Fig. 7I,J). Taken together, our findings indicate that xMIDs play a fundamental role in the remodeling of multiple epithelial tissues.

DISCUSSION

Role of microtubule regulation by xMIDs in cell shape changes and maintenance during neural tube closure

Here, we demonstrated that the xMIDs are required for normal neural tube closure, a multi-step event that involves neural specification, cell proliferation and morphogenetic movements...
In particular, a collective cell movement, which is based on the morphogenesis of cells in the neural plate, serves as the major driving force for its invagination (Colas and Schoenwolf, 2001; Pilot and Lecuit, 2005; Quintin et al., 2008). In xMID morphants, the neuroepithelial cells remained rounded, and the localization of adhesive molecules was perturbed, indicating that the epithelial organization was not maintained. The rearrangement and assembly of microtubules was also impaired in the xMID morphants. The prevention of NTDs in xMID morphants by the expression of another microtubule-associated protein suggests that the primary function of xMIDs is to stabilize microtubules. From these data, we propose that the xMIDs regulate cellular morphogenesis and epithelial organization during neural tube closure through the assembly and stabilization of microtubules. Since the knockdown of the xMIDs did not cause any obvious defects in classical microtubule function in mitosis or primary cilium formation, the impact of xMID knockdown on microtubules might be limited, affecting only their rearrangement and assembly along the apicobasal axis.

Since the cellular and molecular mechanisms of neural tube closure in amphibians are closely related to those in amniotes (Davidson and Keller, 1999), MIDs are probably required for neural tube closure in amniotes, including humans. However, NTDs, such as anencephaly and spina bifida, have not been reported in OS patients, although the expression of human MID1 in the developing neural tube has been reported (Pinson et al., 2004), and abnormalities of the brain, including agenesis or hypoplasia of the cerebellar vermis and corpus callosum, are seen in OS (Fontanella et al., 2008; So et al., 2005). The overlapping expression of MID1 and MID2 in developing neural tissues (Buchner et al., 1999; Granata et al., 2005; Dal Zotto et al., 1998) and the finding that MID1 and MID2 have redundant activities in...
chick left-right determination (Granata et al., 2005), suggest that MID1 and MID2 have redundant functions in neural tube closure such that their role in this process is not unveiled by the knockdown or mutation of only one of them.

**xMID-Mig12 collaboration is required for the rearrangement and stabilization of microtubules in vivo**

*Mig12* was identified as a gene encoding a 152 amino acid protein that is expressed in gastrula-stage zebrafish (Conway, 1995). In Cos7 cells, Mig12 colocalizes with MID1 and stabilizes microtubules, suggesting that Mig12 might function cooperatively with the xMIDs. In this study, we showed that Mig12 cooperates functionally with the xMIDs in regulating microtubule organization during neural tube closure. However, all our data support the idea that the xMIDs function as the dominant regulators of neural tube closure. The strongest evidence for this is that the NTDs of Mig12 morphants were rescued by xMID expression, whereas those of xMID morphants were not rescued by Mig12. Furthermore, the cytoplasmic localization of Mig12-GFP was not changed by the gain or loss of xMID function. These data all suggest that, at least in the neuroepithelial cells, the xMIDs are the main players in the xMID-Mig12 complex, and Mig12 might be recruited in a limited amount to finely modulate the xMID activities. Furthermore, the functional interaction of these proteins might be dynamic and tightly regulated in time and space to avoid overstabilization of the microtubules, which might lead to defects in cellular morphogenesis. Since a regulatory subunit of protein phosphatase 2A (PP2A) binds MID1 and MID2 (Liu et al., 2001; Short et al., 2002; Trockenbacher et al., 2001), it is possible that the PP2A complex is involved in this mechanism.

**Molecular link between microtubules and apical constriction**

The molecular mechanisms governing cellular morphogenesis in epithelia are well documented, especially with regard to apical constriction in *Drosophila* (Dawes-Hoang et al., 2005; Kolsch et al., 2007; Nikolaidou and Barrett, 2004; Pilot and Lecuit, 2005;
Quintin et al., 2008). In vertebrates, Shroom3-mediated activation of ROCKs and myosin II plays a crucial role in driving apical constriction (Haigo et al., 2003; Hildebrand, 2005; Nishimura and Takeichi, 2008; Rolò et al., 2009). Our study of the xMIDs raises the important question of how microtubules control apical constriction in neuroepithelial cells. In Drosophila, RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner (Rogers et al., 2004), and inhibition of microtubule polymerization prevents apical actin assembly and Myosin light chain phosphorylation, thus blocking apical constriction (Corrigall et al., 2007). In addition, the enhancement of cadherin-based cell adhesion is dependent on microtubules in human epithelial cells (Meng et al., 2008). These data suggest that the molecular link between microtubules and apical constriction is mediated by the transport of key regulators of actin polymerization, Myosin II activation, and/or cell-cell contacts. It will therefore be intriguing to examine whether intracellular transport is affected in xMID morphants.

**Insights into the molecular and pathological mechanisms of Opitz G/BBB syndrome**

We demonstrated that xMID1 is required for the morphogenesis of epithelial organs, such as the cement gland, pronephros and foregut. Furthermore, in the xMID morphants, the epithelial cell morphology and organization were severely affected, and the distribution of laminin in the basal lamina was compromised. These data indicate that the morphogenetic defects in xMID morphants are due to the loss of epithelial integrity.

In OS patients, various developmental abnormalities, including craniofacial, urogenital, gastrointestinal and cardiovascular defects are observed, although the pathological mechanisms have not been identified (Fontanella et al., 2008; So et al., 2005). Importantly, a recent analysis of the MID1 expression pattern in the human embryo revealed it to be expressed in various epithelial tissues, including the central nervous system, kidney primordia, and the pharyngeal, respiratory and gastrointestinal epithelia (Pinson et al., 2004). In addition, MID1 is expressed in the anal folds and genital tubercle (Pinson et al., 2004). These expression patterns indicate a strong correlation between epithelial MID1 expression and the development of organs affected by OS. Although no extensive histological characterizations of tissues from OS patients have been reported, there are notable similarities in the pathological features of OS patients and the epithelial defects of xMID morphants. In addition, Mid1 shows similar epithelial expression patterns in mouse (Dal Zotto et al., 1998) and chick (Richman et al., 2002). We propose that common mechanisms underlie the normal mouse (Dal Zotto et al., 1998) and chick (Richman et al., 2002).

References


**Supplementary material**

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**Competing interests statement**

The authors declare no competing financial interests.


