The Nf2 tumor suppressor regulates cell–cell adhesion during tissue fusion

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Tissue fusion, the morphogenic process by which epithelial sheets are drawn together and sealed, has been extensively studied in Drosophila. However, there are unique features of mammalian tissue fusion that remain poorly understood. Notably, detachment and apoptosis occur at the leading front in mammals but not in invertebrates. We found that in the mouse embryo, expression of the Nf2 tumor suppressor, merlin, is dynamically regulated during tissue fusion: Nf2 expression is low at the leading front before fusion and high across the fused tissue bridge. Mosaic N2Z mutants exhibit a global defect in tissue fusion characterized by ectopic detachment and increased detachment-induced apoptosis (anoikis). By contrast with core components of the junctional complex, we find that merlin is required specifically for the assembly but not the maintenance of the junctional complex. Our work reveals that regulation of Nf2 expression is a previously unrecognized means of controlling adhesion at the leading front, thereby ensuring successful tissue fusion.

neurofibromatosis type 2

Tissue fusion is the morphogenic process by which epithelial sheets are drawn together and sealed to form a continuous layer. Failure of tissue fusion is common during human embryogenesis and the consequences can be severe. For example, neural tube defects (NTDs) that disrupt either the closing of the cranial neural tube (prospective brain) or spinal neural tube (prospective spinal cord) affect 3,000 pregnancies per year in the United States and cause life-threatening neurologic deficits (1). Failure of tissue fusion is common during human embryogenesis, where the last major morphogenic event is a tissue closure; tEM, transmission electron microscopy; E, epidermal; ALJC, apico-lateral junctional complex; TJ, tight junction; AJ, adherens junction; tEM, transmission electron microscopy; En, embryonic day n.

Results

Dynamic Regulation of Nf2 Expression at the LF During Tissue Fusion. Nf2 is expressed widely during embryogenesis and in early postnatal life but becomes more restricted in the adult (15), suggesting that merlin may serve a particularly important function in vivo? Is merlin required for the assembly and/or maintenance of the junctional complex? And, is merlin’s affect on junctional complex stability related to its other molecular functions?

In this study, we find that regulation of Nf2 tumor suppressor, merlin, is a novel means of controlling adhesion at the LF during tissue fusion. We provide in vivo evidence that merlin, unlike core components of the junctional complex, is required specifically for the assembly but not the maintenance of the junctional complex (TJs and AJs). This work provides a framework for understanding the unique aspects of mammalian tissue fusion and the role of merlin in development and cancer.

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cently, Akhmametyeva and coworkers generated transgenic through E18.5 (supporting information (SI) Fig. 4). mhp, median hinge point. E probe shows no staining of the VZ (E15.5) (D), marks the dorsal midline where the neural folds have fused. Bracket in D demarcates the junctional zone where eyelid fusion occurred. A control sense probe shows no staining of the VZ (E15.5) (E), mhp, median hinge point.

Fig. 1. Dynamic regulation of Nf2 expression at the LF during tissue fusion. In situ hybridization of frozen sections using an antisense probe that recognizes the Nf2 mRNA shows decreased Nf2 expression in the dorsal NE cells at the tips of the forebrain neural folds at E9.25 (A, arrowheads) and in the peridermal and epidermal cells at the LF of the developing eyelids at E15.5 (C, bracket). After completion of neural tube closure (forebrain vesicle, E10.5) and eyelid fusion (E15.5), the NE cells (B) and epidermal cells (D) that form the respective tissue bridges show strong Nf2 expression. The arrowhead in B marks the dorsal midline where the neural folds have fused. Bracket in D demarcates the junctional zone where eyelid fusion occurred. A control sense probe shows no staining of the VZ (E15.5) (E), mhp, median hinge point.

Deletion of Nf2 in the Embryo by Asynchronous NesCre1p-Mediated Recombination. Nf2−/− mouse embryos die early in gestation (E6.5–7) due to an extraembryonic defect (17), thus the consequences of Nf2 loss in the embryo proper remain largely unknown. To test whether merlin was required for tissue fusion, specifically neurulation, we combined a conditional loss-of-function allele of Nf2 (Nf2flox−/−) with a Cre recombinase transgene expressed from the nestin promoter (NesCre1p) (18, 19). We and other groups have observed different levels of Cre-mediated recombination from the NesCre1p transgene depending on whether the transgene is inherited from the father or mother, presumably due to imprinting effects (20) (SI Fig. 5). The experiments described in this manuscript were performed by using Nf2flox−/− embryos with paternally inherited NesCre1p (designated NesCre1p); we refer to these embryos below as “NesCre1p Nf2 mosaics.”

The NesCre1p transgene provided an ideal system in which to study neurulation, as NesCre1p-mediated recombination could be detected in scattered NE cells at E8.5, before neural tube closure (SI Fig. 6A). By late gestation (E18.5), virtually all of the cells in the brains of NesCre1p-positive mice had undergone recombination (SI Fig. 6B). Near complete NesCre1p-mediated recombination in the brains of late gestation NesCre1p Nf2 mosaics was confirmed by PCR (SI Fig. 6C) and Western blotting for merlin (SI Fig. 6D). This data demonstrated that NesCre1p-mediated recombination was asynchronous, with a subset of NE cells losing Nf2 expression early in midgestation and the remainder of NE cells and their progeny losing Nf2 expression in mid-to-late gestation. The asynchrony of NesCre1p-mediated recombination provided us with the unique opportunity to compare the role of merlin in the assembly versus the maintenance of the junctional complexes within the neuroepithelium.

Global Tissue Fusion Defect in NesCre1p Nf2 Mosaics. Although the vast majority of the NesCre1p Nf2 mosaics survived until the time of birth (SI Table 1), these embryos exhibited a striking defect in tissue fusion. We observed a spectrum of NTDs, including encephalocele, exencephaly and rarely completely open neural tubes (Fig. 2 A–C). Intriguingly, we recovered 8.6 times more embryos with exencephaly at E18.5 (17 exencephalic embryos/142 total embryos including all genotypes) than at E9.5 (2 exencephalic embryos/146 total embryos including all genotypes), suggesting that the major mechanism of exencephaly was neural tube reopening. The cause of reopening may be an accumulation of Nf2-deficient cells defective in cell–cell adhesion and apoptosis (see below).

In addition to NTDs, we observed three tissue fusion defects in the eye (retinal coloboma, lens herniation, and open-eyelid-at-birth) (Fig. 2 D–F), cleft palate (Fig. 2G), omphalocele (Fig. 2H), and cardiac ventricular septal defects (Fig. 2I). All tissue fusion defects had incomplete penetrance (SI Table 2). Defects in chorioallantoic fusion were not detected. In the mouse, this tissue fusion event occurs at E8.5, a time when NesCre1p-mediated recombination is only present in scattered cells in the embryo. A variety of other defects affecting tissue organization and differentiation were also identified in the NesCre1p Nf2 mosaics (SI Figs. 7 and 8).

Merlin Prevents Ectopic Detachment by Promoting the Formation of Apico-Lateral Junctional Complexes. To understand why tissue fusion was failing in the NesCre1p Nf2 mosaics, we studied the NTDs in detail. We compared NesCre1p Nf2 mosaics to developmentally matched wild-type embryos (Fig. 2 J and L), as morphogenetic progression causes marked changes in the structure of the neural tube and frequency of apoptosis, making age-matched controls uninformative. Histologic examination of transverse sections through the brains of E9.5 NesCre1p Nf2 mosaics revealed that NE cells were detaching from the apical surface. In mutants with closed neural tubes, the detached NE cells accumulated within the ventricular space (Fig. 2K). Along the dorsal neural tube where tissue fusion occurred, the NE cells and epidermal ectoderm appeared disorganized, likely predisposing the embryo to neural tube reopening. Severely affected mutants showed widespread detachment of NE cells and cells from the epidermal ectoderm (Fig. 2M). In these mutants, there were likely too few cells remaining for neurulation to proceed. Histologically, the majority of detaching epithelial cells appeared viable: completely detached cells undergoing mitosis were identified (Fig. 2K, upper inset).

Ultrastructural examination of the neuroepithelium revealed the cause of ectopic detachment. By transmission electron
microscopy (tEM), we found areas of neuroepithelium in *NesCre1p Nf2* mosaics in which the apico-lateral junctional complexes (ALJCs) were absent (Fig. 2P). Some NE cells in these areas were detached from the apical surface. Whereas some detached NE cells appeared viable, others showed signs of apoptosis, such as numerous cytoplasmic vacuoles. The remainder of the neuroepithelium in the *NesCre1p Nf2* mosaics had normally distributed ALJCs (Fig. 2O), consistent with the mosaic pattern of *NesCre1p* activity at this stage in development. Thus, merlin is required in vivo for the assembly of ALJCs in the developing nervous system.

We investigated the relationship among *Nf2* expression, detachment, and apoptosis using immunohistochemistry against the apoptotic marker, cleaved caspase-3 (CC3). As reported (21), apoptotic cells in the neuroepithelium of E8.5 controls were predominantly localized to the tips of the neural folds (SI Fig. 9D). Within severely affected E9.5 mutants, the level of apoptosis was increased 38.7-fold compared with the developmentally matched E8.5 controls (*t* test *P* = 0.0005) (Fig. 2Q). Furthermore, apoptotic cells were not restricted to the tips of the neural folds but were found throughout the neural plate (SI Fig. 9E). 91.2% of NE cells that had detached in the *NesCre1p Nf2* mosaic embryos were negative for CC3, consistent with detachment preceding and triggering apoptosis, a phenomenon known as anoikis. Immunohistochemistry for the proliferation marker Ki-67 revealed a similar, very high rate of proliferation within the neuroepithelium of E8.5 controls and severely affected E9.5 mutants (SI Fig. 9A–C). Together, these findings suggest that deletion of *Nf2* inhibits the formation of ALJCs, leading to ectopic detachment and anoikis of NE cells. Furthermore, reduced levels of *Nf2* at the LF may promote detachment and apoptosis at this site during the normal process of tissue fusion.

**Analysis of Merlin Using a Functional Cell–Cell Adhesion Assay.** We demonstrated in vivo that merlin is required for the assembly of ALJCs in the neuroepithelium. To quantify this effect, we used a hanging drop assay that measures the ability of single cells to form cell aggregates and the resistance of these aggregates to a shearing force. MDCK epithelial cells were selected for this experiment because the majority of tissue fusion events involve epithelial cells, and the use of these cells in the hanging drop assay is well established. MDCK cells were transfected with either an empty vector expressing GFP (control) or a vector coexpressing a dominant-negative form of *Nf2* and GFP. Pure populations of GFP-positive cells were isolated by FACS analysis. Overexpression of dominant-negative merlin was confirmed by Western blotting (SI Fig. 10A).

At the beginning of the experiment, all of the cells in the...
hanging drops were present as single cells (SI Fig. 10 B–D). In the control (SI Fig. 10 B and D), the number of cells in large clusters (>25 cells) increased to 31% at 2 h, and to 64% at 4 h. Resistance to trituration increased from 0% of cells remaining in large clusters at 2 h, to 42% of cells at 4 h. Cells expressing dominant-negative Nf2 formed large clusters of >25 cells more slowly, with only 11% of cells in large clusters at 2 h (SI Fig. 10 C and D). Furthermore, the cells expressing dominant-negative Nf2 were more sensitive to trituration. At 4 h, 66% of these cells remained unaffected (SI Fig. 12). The hanging drop assay is the first direct functional test of merlin’s role in adhesion.

**Merlin Is Required for the Assembly but Not Maintenance of Apico-Lateral Junctional Complexes.** After neural tube closure, the NE cells and NE-derived radial glia that line the ventricles [ventricular zone (VZ) cells] do not assemble de novo ALJCs (22, 23). Instead, inheritance of existing ALJCs is determined by the orientation of cleavage during cell division. Electron and confocal microscopy show that a horizontal cleavage produces an apical daughter cell that inherits the entire ALJC and a basal daughter that is specified to become a migratory neuron. By contrast, in a vertical division the cleavage furrow progresses from the basal toward the apical cell surface, where it bisects the existing ALJC between the two daughter cells. Even during mitosis when the VZ cells round up at the ventricular surface, the ALJCs are retained. Therefore, the neuroepithelium before and after neural tube closure offers a unique setting in which the requirement for merlin in ALJC assembly versus maintenance can be assessed in vivo.

In the subset of NesCre1p Nf2 mosaics in which the neural tube closed and remained closed, we examined the VZ in late gestation and found that it had a biphasic appearance. There were discrete clumps of disorganized, loosely attached VZ cells that protruded into the ventricular space, surrounded by well organized, pseudostratified VZ cells (Fig. 3 A–D). These large clumps of disorganized, loosely attached VZ cells, surrounded by well organized VZ (SI Fig. 13), the number of cells in large clusters is confirmed by immunofluorescence for the TJ component ZO-1 and the AJ components, N-cadherin and β-catenin, further confirmed the lack of ALJCs (Fig. 3 D–F). Although present, the apical band of actin was fragmented across the surface of the clumps (Fig. 3G).

Interestingly, tEM demonstrated the presence of ALJCs composed of distinct TJs and AJs within the well organized VZ (Fig. 3 B and C). By immunofluorescence, ZO-1, N-cadherin, β-catenin, and actin were properly localized (Fig. 3 D–F). In fact, TJs were more prominent in the mutant VZ than in the control, possibly reflecting a delay in differentiation (24). Thus, despite the fact that by late gestation there was very little if any merlin remaining in the brains of the NesCre1p Nf2 mosaics, the ALJCs in the well organized regions of the VZ were retained.

To verify that merlin was absent in the well organized regions of the VZ, we took three approaches. First, X-gal staining of NesCre1p Nf2 mosaics carrying the lox-STOP-lox LacZ reporter demonstrated blue staining in the disorganized clumps and in the well-organized VZ, indicating Cre-mediated recombination had occurred (SI Fig. 13A). Second, we performed laser capture microdissection followed by PCR on the well organized VZ and found complete recombination of the Nf2loxp allele to the Nf2lox form (SI Fig. 13B). Third, immunohistochemistry with an anti-merlin antibody confirmed the absence of merlin in the mutant VZ (SI Fig. 13D).

This data in combination with the NesCre1p reporter analysis showing asynchronous recombination indicates that NE cells that lose Nf2 early in gestation fail to assemble ALJCs and are prone to detaching, whereas NE cells and NE-derived radial glia that lose Nf2 later in gestation are able to maintain existing ALJCs. This finding that merlin is required for the assembly but not the maintenance of ALJCs is completely unexpected based on the prior in vitro studies and contrasts sharply with the phenotype of mouse embryos in which core components of the junctional complex are deleted. Asynchronous deletion of β-catenin in the developing forebrain (between E8.75 and E10.5), for example, results in loss of the entire forebrain and
anterior facial structures, not exencephaly (25, 26). AJs are completely absent from the NE cells of these FoxG1Cre;β-cateninfl/fl embryos at E9.5, and there is massive detachment of NE cells into the ventricular space, consistent with a requirement for β-catenin in assembly and maintenance of the AJs.

Discussion
Successful morphogenesis in the mammalian embryo requires the completion of multiple tissue fusion events. In contrast to dorsal closure in Drosophila, mammalian tissue fusion is characterized by detachment and apoptosis at the LF. Little is known about the physiologic role of these processes and how they are controlled. In this report, we show that the expression of the NF2 tumor suppressor, merlin, is dynamically regulated during tissue fusion, with decreased levels at the LF before fusion and high levels across the fused tissue bridge.

The upstream pathways that regulate NF2 expression remain to be elucidated. Both BMP and Wnt/β-catenin signaling pathways are attractive candidates to regulate NF2 transcription, as mutations in either pathway can lead to NTDs in mice (27, 28). Several BMP ligands and targets of BMP signaling are expressed in dorsal neural tissue, where NF2 levels are transiently decreased. During brain development, Wnt/β-catenin signaling up-regulates expression of the Sp5 gene, a member of the Sp1 transcription factor family that has the same DNA binding specificity as Sp1 but represses Sp1 target genes (29). Site-directed mutagenesis studies of the NF2 promoter indicate that there is a GC-rich sequence (position −58 to −46) that can be bound by Sp1 (30), and possibly may be repressed by Sp5.

Merlin, in turn, regulates the assembly of ALJC, acting as a developmental switch to loosen and tighten cell–cell adhesion. We provide evidence that loosening of cell–cell adhesion (detachment) may serve as a trigger of apoptosis at the LF, and propose that detachment-induced apoptosis (anoikis) may ensure that only those epithelial cells that form stable cell–cell contacts survive to contribute to the tissue bridge. As merlin has been previously shown to associate with β1-integrin complexes (31), it is also possible that loosening of cell-matrix interactions may contribute to detachment and anoikis.

We find that merlin is required in vivo for the assembly but not maintenance of ALJC. The process of junctional complex assembly has been studied extensively in MDCK epithelial cells. In this model system, transinteractions between E-cadherin molecules initiate adhesion between adjacent cell membranes (32). This is followed by the formation of a primordial junction composed of AJ and TJ components, which matures into a distinct TJ and AJ. Remodeling of cortical actin and localized lamellipodial activity occur early in the process of initiating cell–cell contacts and correlate with the spatiotemporal regulation of Rac: Rac transiently concentrates at the newest sites of contact and decreases at older, stabilized sites (33). Thus, one model is that merlin might regulate junctional complex assembly by inhibiting Rac-dependent signaling. However, there was no increase in the Rac effector phospho-c-Jun either at the LF in normal embryos nor in mutant embryos (M.E.M. and T.J., data not shown), suggesting that decreased NF2 expression does not lead to a dramatic increase in Rac-dependent signaling in this developmental context. It is possible that merlin inhibits Rac in specific subcellular compartments, such as the site of cell–cell contact. Merlin may also regulate junctional complex assembly through regulation of the actin cytoskeleton or receptor density at the cell surface, as both actin and signaling receptors are intimately associated with the ALJC (34, 35). As merlin is dispensable for the maintenance of the ALJC, it is unlikely that merlin serves as a core structural component.

Our finding that merlin is required for the assembly but not maintenance of ALJC has important implications for the role of NF2 loss in tumorigenesis. If NF2 loss occurs in a nondividing cell with mature junctional complexes, there may be no adverse consequences. However, if NF2 loss occurs in a dividing cell or a nondividing cell that at a later point in time reenters the cell cycle and needs to assemble junctional complexes de novo (e.g., during tissue repair), then tumorigenesis may be initiated: cells that cannot form ALJC and establish apicobasal polarity will be unable to form a well-organized tissue and will be resistant to contact-dependent growth arrest. Notably, it has long been recognized that the risk for developing a meningioma, a tumor that frequently harbors NF2 mutations, is significantly increased in patients with a history of head trauma (36, 37). Thus, the elucidation of the consequences of NF2 deficiency in the developing embryo may provide a framework to understand how germ-line and sporadic mutations of this tumor suppressor contribute to tumor formation.

Materials and Methods
SI Methods provide further details.

RNA In Situ Hybridization. We used a modification of the methods described by Wilkinson and Nieto to perform in situ hybridization on 15-μm frozen sections of E8.5-E18.5 embryos (38). The primers 5′-TTACTATTAACCCACTTG-3′ and 5′-CGCTTCTATGCGGATCC-3′ were used to PCR amplify an 836-bp fragment from the C-terminal half of the NF2 cDNA. This fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA), creating pMB84. By using primers to the Sp6 promoter and the T7 promoter, linear DNA fragments containing the 836-bp NF2 fragment were generated by PCR amplification. An antisense digoxigenin riboprobe for NF2 was generated by incubating the linear DNA fragments with Sp6 RNA polymerase, and a sense digoxigenin riboprobe for NF2 was generated by incubating the linear DNA fragments with T7 RNA polymerase.

tEM. Whole E8.5 and E9.5 embryos or eyes and surrounding soft tissue from E15.5 embryos were placed into fixative consisting of 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer with 0.08 M CaCl2 for 24 h at 4°C. Specimens were processed and viewed as described (39).

Mice and Generation of Embryos. NesCre1 transgenic mice were obtained from Andreas Trumpp (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) and Nf2flox2/flox2 mice were obtained from Marco Giovannini (Foundation Jean Daussset-Centre d’Etude du Polymorphisme Humain, Paris, France). NesCre1 mice were crossbred to Nf2flox2/flox2 males. Male offspring expressing Cre were crossed to Nf2flox2/flox2 females. NesCre1 mice were separately crossed to lox-STOP-lox LacZ reporter mice Gt(Rosa)26Sortm1Sor (Jackson Laboratories, Bar Harbor, ME) to generate offspring with paternally or maternally inherited Cre. NesCre1/Nf2flox2+ male mice were also crossed to Nf2flox2/flox2, Gt(Rosa)26Sortm1Sor female mice to generate mutant embryos containing the lox-STOP-lox LacZ reporter. Details of PCR genotyping reactions are available upon request. The morning of plug detection was taken as E0.5, and embryos were collected from mid-gestation to birth. Embryos were dissected from the mother, either the yolk sac or the tail was collected for genotyping, and embryos were fixed overnight in Bouin’s fixative, 10% neutral buffered formalin (NBF), or 4% paraformaldehyde (PFA) in PBS. Embryos were processed and embedded in paraffin, and 4-μm sections were cut. Research was conducted in compliance with the Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the

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Immunohistochemistry and Immunofluorescence. Whole E8.5 and E9.5 embryos or brains from E15.5 and E18.5 embryos were fixed in 10% NBF for 1 h or 24 h, respectively. For detecting merlin by immunohistochemistry, brains from E18.5 embryos were snap-frozen. Seven-micrometer sections were cut and fixed in acetone at 4°C for 10 min. We used a modification of the methods described by M.E.M and J.T. to perform immunohistochemistry and immunofluorescence (40). The following antibodies were used: Ki-67 (Novocastra, Newcastle, United Kingdom; 1/200), CC3 (Cell Signaling, Beverly, MA; 1/100), merlin (Cell Signaling; #9168, 1/100), ZO-1 (Zymed Laboratories, South San Francisco, CA; 1/50), N-cadherin (BD Transduction Laboratories, San Jose, CA; 1/100), β-catenin (Cell Signaling; 1/100), and β-actin (Sigma, St. Louis, MO; 1/50). Ki-67 and CC3-positive cells and tissue areas were determined by using Bioquant Image Analysis software in manual measurement mode.

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