Apoptosis is not required for mammalian neural tube closure

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Apoptotic cell death occurs in many tissues during embryonic development and appears to be essential for processes including digit formation and cardiac outflow tract remodeling. Studies in the chick suggest a requirement for apoptosis during neurulation, because inhibition of caspase activity was found to prevent neural tube closure. In mice, excessive apoptosis occurs in association with failure of neural tube closure in several genetic mutants, but whether regulated apoptosis is also necessary for neural tube closure in mammals is unknown. Here we investigate the possible role of apoptotic cell death during mouse neural tube closure. We confirm the presence of apoptosis in the neural tube before and during closure, and identify a correlation with 3 main events: bending and fusion of the neural folds, postfusion remodeling of the dorsal neural tube and surface ectoderm, and emigration of neural crest cells. Both *Casp3* and *Apaf1* null embryos exhibit severely reduced apoptosis, yet neurulation proceeds normally in the forebrain and spine. In contrast, the mutant embryos fail to complete neural tube closure in the midbrain and hindbrain. Application of the apoptosis inhibitors z-Vad-fmk and pifithrin-α to neurulation-stage embryos in culture suppresses apoptosis but does not prevent initiation or progression of neural tube closure along the entire neuraxis, including the midbrain and hindbrain. Remodeling of the surface ectoderm to cover the closed tube, as well as delamination and migration of neural crest cells, also appear to be normal in the apoptosis-suppressed embryos. We conclude that apoptosis is not required for neural tube closure in the mouse embryo.

cell death | embryo | morphogenesis | neurulation

Formation of the neural tube through the process of neurulation is a critical event in embryogenesis, the neural tube being the precursor of the entire central nervous system. Neural tube closure involves a complex sequence of morphogenetic movements, involving elevation and bending of the neural folds, adhesion and fusion of the apposing fold tips, and tissue remodeling to form a continuous neuroepithelial tube with overlying surface ectoderm (1). Failure of closure results in neural tube defects (NTDs), including exencephaly (in the cranial region) and spina bifida (resulting from failure of spinal closure) (2).

The high frequency of NTDs in humans (1 per 1,000 established pregnancies) and in more than 190 genetic mutant mice suggests that neurulation is a sensitive and complex process. Numerous gene functions are required for successful completion of neural tube closure (2, 3), and different molecular pathways are needed for various phases of the closure process. For example, planar cell polarity signaling is required for shaping of the neural plate and initiation of closure (2, 4), whereas bone morphogenetic protein signaling plays an important role in regulation of neural fold bending (5).

Although many genes have been implicated in neural tube closure, the cellular mechanisms underlying closure are much less well understood. One hallmark of the neurulation process is the presence of dying cells in the neural folds during and after closure. Cell death in the neuroepithelium was first documented by ultrastructural studies, and dying cells were subsequently found to exhibit the characteristic features of apoptosis (6–8). But despite having first been noted more than 30 years ago, the physiological role of cell death in the neuroepithelium during mammalian neural tube closure remains to be established.

Experimental evidence of a requirement for apoptosis in neurulation has come from findings that neural tube closure in chick embryos fails after suppression of apoptosis by in ovo treatment with the pan-caspase inhibitor z-VAD-fmk (9). In mice, several knockout strains exhibit alterations in the abundance of apoptotic cells during development of NTDs. Whereas most of these strains exhibit increased cell death, a few genetically determined mouse NTDs have been associated with diminished apoptosis (2, 3). This appears to support a requirement for apoptosis in mouse neural tube closure, although definitive evidence for this hypothesis is lacking. To resolve this question, we decided to investigate experimentally the requirement for apoptosis in mouse primary neurulation.

Results

Patterns of Cell Death During Neural Tube Closure. In mouse embryos, apoptotic cells are particularly evident in the rostral forebrain, the midline of the closed neural tube, and the rhombomeres (10, 11). If regulated apoptosis were in fact required for neurulation, then the closing neural folds should exhibit characteristic patterns of apoptosis; however, this has not been systematically investigated. We determined the spatiotemporal distribution of apoptotic cells by whole-mount TUNEL staining and immunostaining for activated caspase-3 at sequential stages throughout mouse neural tube closure from embryonic day (E) 8.5 to E10. This analysis revealed that apoptosis occurs predominantly in spatial and temporal correlation with 3 main events: bending and fusion of the neural folds, postfusion remodeling of the dorsal neural tube and surface ectoderm, and migration of the neural crest cells (NCCs) away from the neural tube (Fig. 1).

Neural Tube Closure Is Completed in Forebrain and Spine of Mutant Embryos Lacking Apoptosis. To investigate whether apoptosis is required for mouse neural tube closure, we examined embryos with targeted genetic mutations that lead to marked reduction or absence of apoptosis. Caspases are a family of cysteine proteases that have a primary function at several steps of the apoptotic cascade (12). Among these proteins, caspase-3 is the major effector caspase that cleaves protein substrates within the apo-

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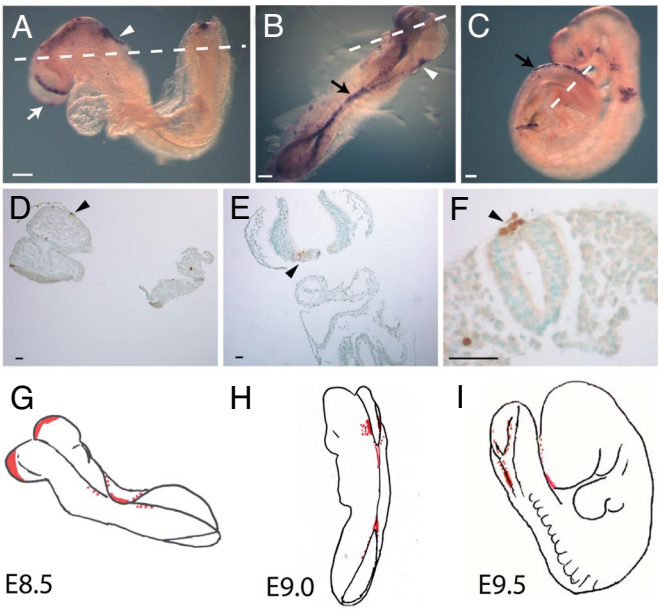

Fig. 1. Spatiotemporal analysis of apoptosis in the neuroepithelium. TUNEL (*A–C*) and activated caspase-3 (*D–F*) staining of CD1 embryos at E8.5 (*A* and *D*), E9.0 (*B* and *E*), and E9.5 (*C* and *F*) reveals the presence of apoptotic cells at several sites in the open neural folds and closed neural tube. Scattered TUNEL-positive (blue) cells are seen throughout the neural folds (*A* and *B*) and are particularly abundant in the forebrain neural folds (arrow in *A*), in a rhombomeric pattern within the hindbrain (arrowheads in *A* and *B*), and in the dorsal midline of the closed neural tube (black arrows in *B* and *C*). The locations of cells positive for activated caspase-3 (brown staining, arrowheads in *D–F*) correspond to sites of abundant TUNEL staining. Representative sections in *D–F* are from levels indicated by dotted lines in *A–C*, respectively. Drawings (*G–I*) summarize the main sites of apoptosis (in red) seen at E8.5 (*G*), E9.0 (*H*), and E9.5 (*I*). At least 8 embryos per stage were analyzed. (Scale bar: 0.1 mm.)

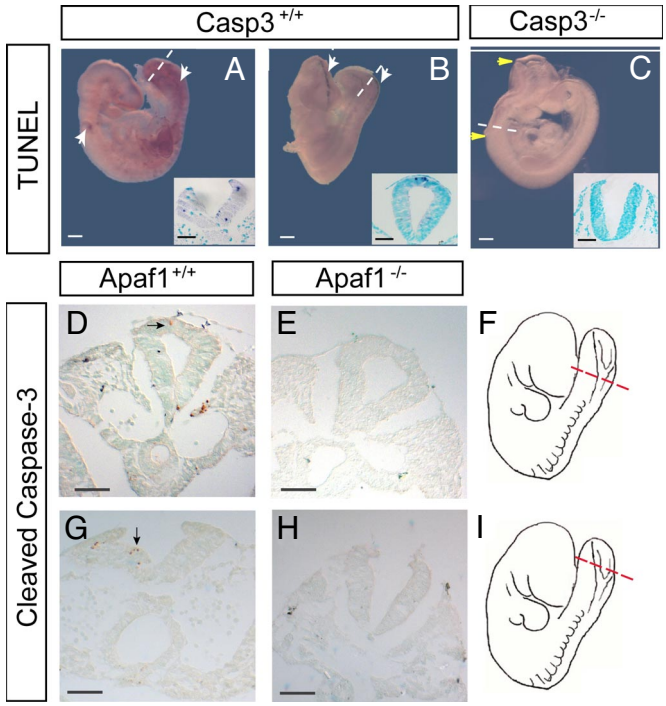

Fig. 2. Genetic inhibition of apoptosis does not prevent neurulation in forebrain or spine. (*A–C*) Whole-mount TUNEL staining of *Casp3* wild-type (*A* and *B*) and null (*C*) embryos. Insets show transverse sections through the spinal region at levels indicated by dashed lines in *A–C*. Despite exposure to identical staining conditions, there is a complete lack of TUNEL-positive cells in mutant embryos (*C*), whereas wild-type littermates exhibit plentiful apoptosis (white arrowheads in *A* and *B*), in a pattern comparable to that seen in CD1 embryos (see Fig. 1). Note that the neural tube has closed in the forebrain and spinal region of the *Casp3*$^{-/-}$ embryo, whereas the caudal midbrain and hindbrain have remained open (i.e., exencephaly, between the yellow arrows). (*D–I*) Transverse sections through the spinal neural tube (at axial levels shown in *F* and *I*) of *Apaf1* wild-type (*D* and *G*) and null (*E* and *H*) embryos immunostained for activated caspase-3. In the mutant embryos, no staining is evident in either closed (*E*) or open (*H*) neural tube, whereas wild-type littermates exhibit immunoreactive cells in the neuroepithelium at both closed (*D*) and open (*G*) neural tube levels. The *Apaf1*$^{-/-}$ embryos exhibit midbrain/hindbrain exencephaly similar to that of the *Casp3*$^{-/-}$ embryos, whereas the neural tube is closed in forebrain and spinal regions (not shown). (Scale bars: 0.1 mm.)

ptotic cell. *Casp3* mutant embryos exhibit exencephaly with variable penetrance depending on genetic background (13). We analyzed apoptosis in *Casp3* null and wild-type embryos at E9.5 by whole-mount TUNEL staining (because immunostaining for activated caspase-3 cannot be performed in null embryos). The pattern of apoptosis in wild-type (C57BL/6J background) embryos (Fig. 2*A* and *B*) was comparable to the CD1 embryos that we used to establish the spatial and temporal occurrence of cell death (Fig. 1). In contrast, *Casp3*$^{-/-}$ embryos were negative for TUNEL staining, as assessed by analysis in both whole mounts and sections (Fig. 2*C*). Despite this lack of apoptosis, neurulation proceeded to completion in both the forebrain and throughout the spinal region of *Casp3*$^{-/-}$ embryos (Fig. 2*C*). In contrast, the mutant embryos exhibited failure of neural tube closure in the caudal midbrain and throughout the hindbrain, as reported previously (13).

We also investigated the relationship between genetic inhibition of apoptosis and neural tube closure in a second model, the *Apaf1* knockout mouse. Apaf-1, the homologue of CED-4/ARK, is required for activation of caspase-9; lack of Apaf-1 results in reduced activation of caspase-3 (12, 14). Immunostaining for activated caspase-3 revealed a complete absence of apoptosis in the open neural folds or closed neural tube at all axial levels of *Apaf1* null embryos (Fig. 2*E* and *H*), in contrast to wild-type littermates, in which apoptotic cells were readily detected in both closed (Fig. 2*D*) and closing neural tubes (Fig. 2*G*). Occasional positive cells were observed in *Apaf1*$^{-/-}$ embryos, but only in such tissues as dorsal root ganglia, where apoptosis is especially abundant (Fig. 2*H*). As reported previously (14), *Apaf1*$^{-/-}$ embryos exhibited midbrain and hindbrain exencephaly (data not shown), whereas the forebrain and spinal regions exhibited normal neural tube closure.

Thus, the analysis of *Casp3* and *Apaf1* mutants demonstrates that, contrary to expectations based on previous chick studies (9), apoptosis is not required for neural tube closure either at the rostral-most (forebrain) level or throughout the spinal region. In contrast, because both mutants exhibit failure of closure in the midbrain and hindbrain, the possibility remains that apoptosis is specifically required for closure at this level of the neuraxis.

Inhibition of Apoptosis In Vitro Does Not Perturb Neural Tube Closure. To complement the genetic approach to analysis of apoptosis in mouse neurulation, we next used the whole embryo culture system (15) to treat neurulation-stage embryos with chemical inhibitors of apoptosis. The pan-caspase inhibitor z-VAD-fmk and the p53 inhibitor pifithrin-α are both known to suppress apoptosis in various systems (16). First, we evaluated the efficacy and duration of action of the inhibitors by culturing embryos for increasing periods and then assessing the suppression of apoptosis by whole-mount TUNEL staining and immunostaining for

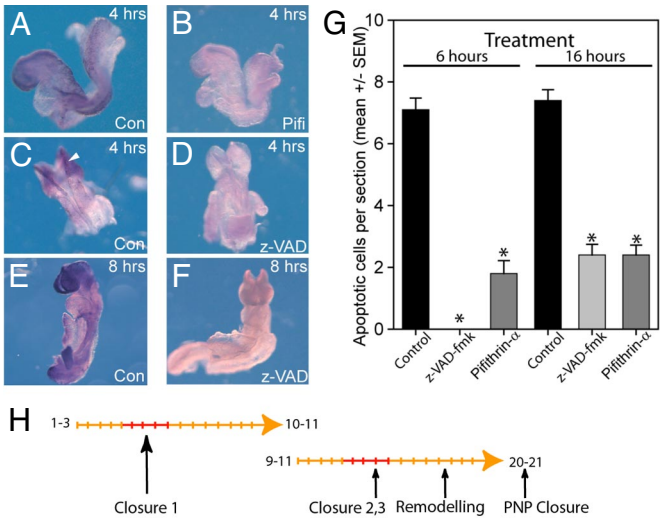

Fig. 3. Pifithrin-α and z-VAD-fmk suppress apoptosis in cultured embryos. (*A–F*) TUNEL staining of DMSO-treated control (*A*, *C*, and *E*), pifithrin-α–treated (*B*), and z-VAD-fmk–treated (*D* and *F*) E8.5 embryos after 4 h (*A–D*) and 8 h (*E* and *F*) of culture. TUNEL-positive cells are clearly visible in the control embryos (e.g., arrowhead in *C*) but are absent from the treated embryos. (*G*) After 6 h of culture, there are no activated caspase-3–positive cells in the spinal neural tube immediately rostral to the site of closure in the z-VAD-fmk–treated embryos, whereas the number of apoptotic cells is significantly reduced in embryos treated with pifithrin-α (dark-gray bar) compared with controls (black bar). After 16 h of culture, the number of activated caspase-3–positive cells in the spinal neural tube rostral to the site of closure remains significantly diminished in embryos treated with z-VAD-fmk (light-gray bar) and pifithrin-α (dark-gray bar) compared with controls (black bar) (*$P < .05$; 1-way ANOVA with Dunn's correction). Ten sections were counted from each of 2 embryos in each experimental group. (*H*) Time line of embryo cultures in the study. Numbers indicate somite stages, and yellow lines (divided into hourly intervals) indicate the duration of cultures designed to evaluate the effect of inhibition of apoptosis on neurulation. Red portions of the lines indicate the stage at which particular events of neural tube closure occur. Thus, closure initiation (closure 1) and the events of cranial neurulation (closures 2 and 3) occurred ≈6–7 h into each culture period, when apoptosis inhibition was maximal. Neural fold remodeling and posterior neuropore closure were assessed ≈12–16 h into the second culture period, when apoptosis was significantly inhibited but not maximal.

activated caspase-3. Apoptosis was completely suppressed by z-VAD-fmk and significantly suppressed by pifithrin-α during a 4-h (Fig. 3*A–D* and *G*) or 8-h (Fig. 3*E–G*) culture period. These effects were observed at doses that had no embryotoxic or growth-retarding effects, as indicated by yolk sac circulation, growth parameters, and morphological appearance (Fig. 3*A–F*; data not shown). Embryos also were cultured overnight for 16 h in the presence of inhibitors to assess whether apoptosis would recover over time. Immunostaining for activated caspase-3 revealed a significant (≈70%) reduction in the abundance of apoptotic cells in embryos exposed for 16 h to z-VAD-fmk or pifithrin-α compared with controls (Fig. 3*G*).

Closure of the neural tube in the mouse is a discontinuous process with initiation sites at the cervical–hindbrain boundary (closure 1), the forebrain–midbrain boundary (closure 2), and the rostral forebrain (closure 3) (1). Progression of closure between these sites results in formation of the neural tube in the cranial region, whereas the spinal neural tube is formed by caudal progression of closure from the site of closure 1, with final closure at the posterior neuropore. To analyze the effects of lack of apoptosis on each of these events, embryo culture was initiated at developmental stages preceding closure at particular sites (Fig. 3*H*; Table 1). Thus, closure 1 is completed after ≈6–7 h in embryos cultured from the 1–3 somite stage. Whereas exposure to either z-VAD-fmk or pifithrin-α during this culture period completely blocked apoptosis (Fig. 3*E–G*), it had no detrimental effect on closure 1 (Table 1). Embryos cultured from the 9–11 somite stage underwent initiation of closure at sites 2 and 3 after 6–7 h, followed by progression to final formation of the cranial neural tube by the 15–16 somite stage (8–10 h into the culture period). The inhibitors had no apparent deleterious effect on completion of any of these cranial neurulation events, with 100% closed brains found in all groups (Table 1). These experiments suggest that apoptosis is dispensable for closure of the entire cranial neural tube, including the midbrain and hindbrain, which remained open in the *Casp3* and *Apaf1* mutants.

Spinal neurulation also appears independent of apoptosis. The length and width of the posterior neuropore after culture were not significantly greater in the treated embryos than controls (Fig. 4*A* and *B*). Moreover, these values decreased progressively with somite stage, as expected (17). We tested whether inhibition of apoptosis may be compensated for by decreased proliferation in the spinal neural folds, resulting in an overall balance of cell number. The mitotic index, as assessed by immunostaining for phospho-histone H3, did not differ significantly between the treatment groups (Fig. 4*C–F*). There appeared to be no compensatory reduction in proliferation in the inhibitor-treated embryos.

Table 1. Initiation and completion of cranial neural tube closure in cultured embryos does not require apoptosis

Experimental group	Somite number at the start of culture	Somite number at the end of culture (± SEM)	Embryos with complete closure
Assessment of closure 1			
Control	1–3	10.42 ± 0.65	7/7 (100%)
Pifithrin-α	1–3	10.71 ± 0.29	7/7 (100%)
z-VAD-fmk	1–3	8.57 ± 0.37	7/7 (100%)
Assessment of closures 2 and 3 and completion of cranial neurulation			
Control	9–11	20.50 ± 0.59	16/16 (100%)
Pifithrin-α	9–11	20.25 ± 0.86	9/9 (100%)
z-VAD-fmk	9–11	19.56 ± 0.52	16/16 (100%)

Embryos were cultured in the presence of DMSO (vehicle control) or apoptosis inhibitors through to the time of initiation of neurulation (closure 1) or the occurrence of cranial neurulation (closures 2 and 3). The culture periods are illustrated in Fig. 3*H*. The number of somites at the end of the culture period was slightly lower in the z-VAD-fmk–treated embryos than in controls, although there was no statistically significant difference between treatment groups at either stage ($P > .05$; 1-way ANOVA).

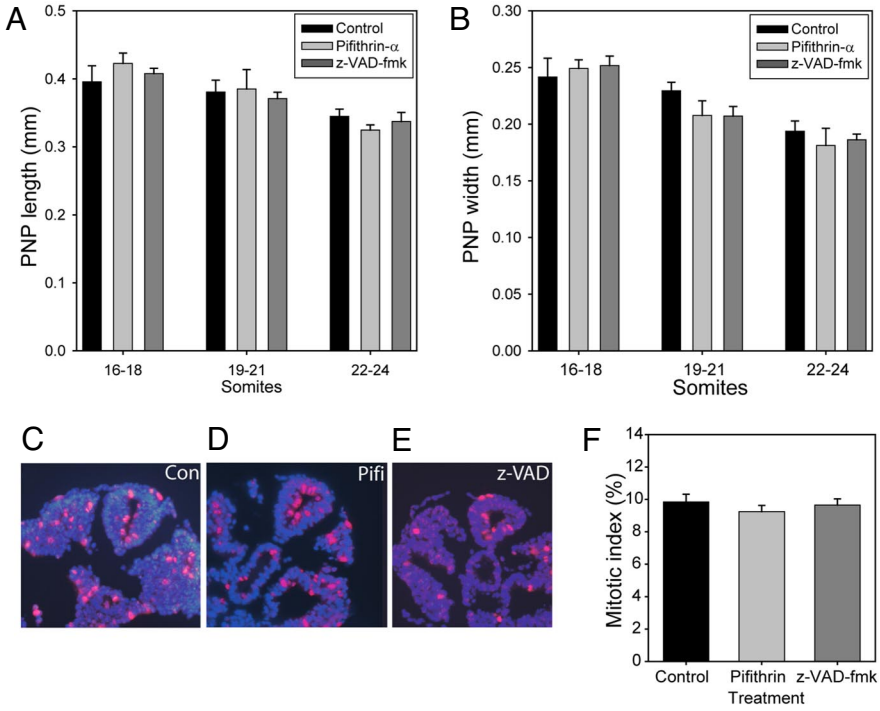

Fig. 4. Inhibition of apoptosis does not adversely affect spinal neurulation. After overnight culture in the presence of apoptosis inhibitors, dimensions of the posterior neuropore indicate the progression of spinal neurulation. No differences in posterior neuropore length (*A*) or width (*B*) were noted between the experimental groups at each somite stage analyzed. Note the progressive decline in both parameters with increasing somite stage. A minimum of 3 embryos per experimental group were assessed at each somite stage. Compared with control embryos (*C*), culture in the presence of pifithrin-α (*D*) or z-VAD-fmk (*E*) had no apparent effect on mitotic activity, as indicated by immunostaining for phospho-histone H3 (pink-stained cells in *C–E*). (*F*) Mitotic index, calculated for the 5 sections rostral to the site of neural tube closure (3 embryos per experimental group), did not differ between treatment groups ($P > .05$; 1-way ANOVA). Graph bars represent mean ± SE.

Postfusion Remodeling and NCC Migration Do Not Depend on Apoptosis. Although our data suggest that apoptosis is not required for adhesion and fusion of the neural folds, we hypothesized that it may play a role in the tissue remodeling process that results in separation of the newly formed neural tube from the overlying surface ectoderm. To investigate this idea, embryos were cultured overnight in the presence of z-VAD-fmk or pifithrin-α, and inhibition of apoptosis was confirmed by immunostaining for activated caspase-3 (Fig. 5*A–C*). Somite matched embryos were sectioned and immunostained for the surface ectoderm marker E-cadherin. Analysis of the site of neural tube closure and the immediately rostral sections, where remodeling occurs, revealed no difference in tissue architecture between the control and treated embryos (Fig. 5*D–I*). Specifically, the midline surface ectoderm was complete in all embryos, despite almost total inhibition of apoptosis. These observations argue against the idea that apoptosis is required for the tissue remodeling necessary to separate the surface ectoderm from dorsal neural tube.

Because TUNEL-positive cells are abundant at sites of NCC migration, we tested whether cell death is required for NCC migration from the tips of the neural folds. After overnight culture of embryos with apoptosis inhibitors, we performed whole-mount in situ hybridization for the NCC marker *cadherin-6*. In embryos cultured under control conditions, *cadherin-6* was expressed in distinct streams of NCC migrating from the hindbrain toward the first and second branchial arches (Fig. 6*A*). Comparable expression patterns were observed in embryos cultured in the presence of pifithrin-α or z-VAD-fmk (Fig. 6*B* and *C*), and the NCC streams remained distinct. Thus, based on this evidence, apoptosis apparently is not required for normal NCC emigration from the hindbrain in mice.

Discussion

In the present study, we observed apoptotic cells in specific locations within the closing neural tube, as described previously (6–8). This supports the hypothesis that apoptosis may have a functional role in neural tube closure, as suggested for chick neurulation (9). Indeed, several targeted mutant mouse strains exhibit both NTDs and altered apoptosis levels (2, 3). For example, NTDs in embryos lacking *ApoB* (18), *Bcl10* (19), *Mdm4* (20), or *Tulp3* (21) function are all associated with increased rates of apoptosis. In these cases, excess apoptosis could result in insufficient numbers of cells to participate in the crucial morphogenetic movements underlying neural tube closure.

In other genetic mutants [e.g., *Trp-53* (22), *Casp3* (23), and *Apaf1* (14) knockouts], NTDs are associated with reduced levels of embryonic apoptosis. We examined this category of mutants in greater detail and found that whereas apoptosis is entirely suppressed in the closing neural tube of both *Casp3* and *Apaf1* null mutants, closure occurs normally in the forebrain and spinal regions. Only in the midbrain and hindbrain of these mutants is closure defective, leading to exencephaly. Strikingly, however, when we cultured neurulation-stage mouse embryos in the presence of chemical inhibitors of apoptosis, we found that even the midbrain and hindbrain close normally in the absence of cell death. Similarly, remodeling of the neuroepithelium and surface ectoderm postfusion proceeded normally. Thus, our findings argue strongly that the reduced apoptosis in the *Casp3* and *Apaf1* mutant mice is unlikely the direct cause of their cranial NTDs, and that other cellular functions likely are disturbed, leading to failure of neurulation.

One difference between genetic and inhibitor-based suppression of apoptosis is the period of developmental time over which apoptosis is absent. In the case of the *Casp3* and *Apaf1* mutants,

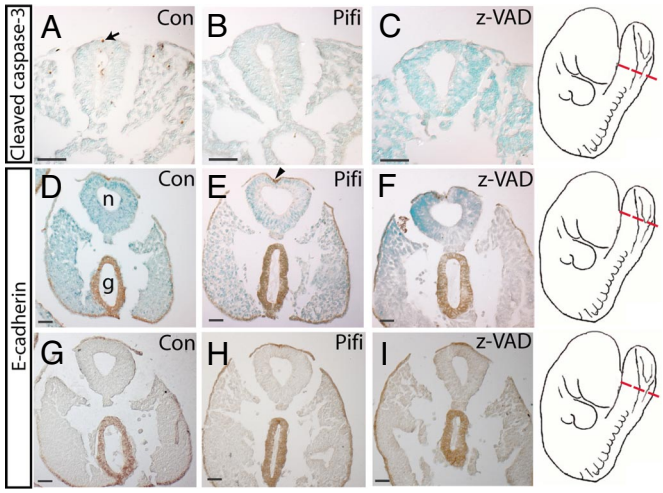

Fig. 5. Apoptosis is not required for neural fold remodeling postfusion. Transverse sections through the spinal neural tube of stage-matched (19 somites) control (*A*, *D*, and *G*), pifithrin-α–treated (*B*, *E*, and *H*), and z-VAD-fmk–treated (*C*, *F*, and *I*) embryos, immunostained for activated caspase-3 (*A–C*) or E-cadherin (*D–I*). Note the lack of apoptosis in the inhibitor-treated embryos (*B* and *C*) ≈12 h into the culture period compared with the control embryos, in which caspase-3–positive cells are seen both in the midline (arrow in *A*) and elsewhere. At an axial level immediately rostral to the site of neural fold fusion, the E-cadherin–positive surface ectoderm (arrowhead in *E*) is already intact in all treatment groups (*D–F*), indicating completion of neural fold remodeling. The midline dorsal indentation indicates the close proximity of the section to the point of neural fold remodeling. The surface ectoderm also is intact at a more rostral level, where remodeling occurred earlier (*G–I*). Breaks in the surface ectoderm lateral to the neural tube represent histological processing artifacts. Abbreviations: g, hindgut; n, neural tube. (Scale bar: 0.1 mm.)

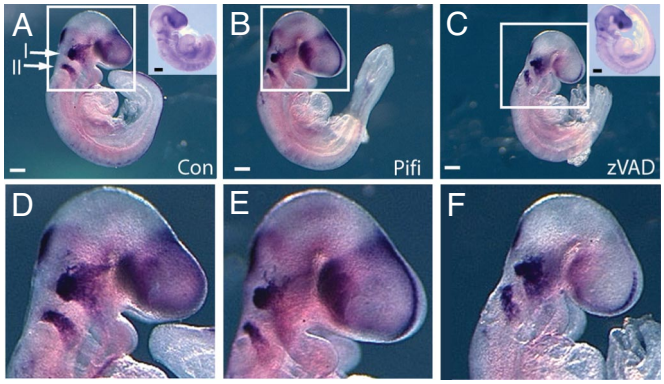

Fig. 6. Neural crest migration from the hindbrain does not appear to be affected by inhibition of apoptosis. Whole-mount in situ hybridization for the neural crest marker *cadherin-6* in control (*A*), pifithrin-α–treated (*B*), and z-VAD-fmk–treated (*C*) embryos reveals no differences in the pattern of NCC emigration. (*D–F*) High-magnification views of the white boxed areas shown in *A–C*. Two distinct streams of NCC (I and II; arrows in *A*) are seen to migrate toward the first and second branchial arches. In the inhibitor-treated embryos (*B*, *C*, *E*, and *F*), the separation of migration streams is retained, and there is no obvious reduction in NCC number. A minimum of 3 embryos per experimental group were analyzed. Because z-VAD-fmk treatment causes slight growth retardation, some embryos were cultured for an additional 4 h, to allow comparison with stage-matched controls (inset in *C*, with concurrently hybridized control shown in the inset in *A*). (Scale bar: 0.2 mm.)

long-term suppression of apoptosis could lead to alterations in the number or distribution of cells in the neural tube, which might be incompatible with closure in the midbrain and hindbrain, although permissive for closure in the forebrain and spine. In contrast, short-term suppression of apoptosis in embryo culture clearly demonstrates that the entire sequence of neural tube closure events can occur in the absence of cell death.

In terms of the overall role of apoptosis in differing development contexts, a requirement for cell death in removal of tissue [as in, e.g., digit separation (24)] is mechanistically easy to explain, and a role in large-scale remodeling (as in, e.g., the cardiac outflow tract) also is plausible (25). A role in tissue fusion is less well defined, however. We find it intriguing that the requirement for apoptosis in another mammalian fusion process, palatal closure, also is controversial. Cell death is abundant in the midline epithelial seam before mesenchymal confluence during fusion of the palatal shelves; however, experimental inhibition of apoptosis during palatal shelf fusion has produced conflicting results, with reports of prevention (26) or no detrimental effect (27) on palatal fusion. Thus, the occurrence of apoptosis at the site of tissue fusion events during development may not indicate a functional role, but rather may be a secondary outcome.

Why do our findings differ from those based on inhibition of apoptosis in chick neurulation (9)? Avian and mammalian neurulation have some fundamental differences (28), raising the question of whether apoptosis may play an obligatory role in neural tube closure in chicks but not in mice. When chick embryos were treated with z-Vad-fmk, the finding of failed neural tube closure was tentatively related to defective remodeling of the neuroepithelium and surface ectoderm (9). For this reason, we specifically assessed tissue remodeling after neural fold fusion in our mouse embryos, but observed no defect in this process in the absence of apoptosis. Interestingly, data from a recent study of chick neurulation (29) show that both neural tube closure and tissue remodeling can occur normally after treatment with z-Vad-fmk. Thus, whether there is actually a fundamental difference between the requirement for apoptosis in avian and mammalian neurulation or whether cell death is dispensable for neural tube closure in both animal groups remains unclear.

In conclusion, we confirm the association of extensive apoptosis with the closing neural tube in mouse embryos, but demonstrate experimentally that this cell death is not essential for completion of neural tube closure.

Experimental Procedures

Mice. The animal studies were carried out under regulations of the UK's Animals (Scientific Procedures) Act 1986 and in accordance with guidance issued by the Medical Research Council in *Responsibility in the Use of Animals for Medical Research* (http://www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC001897). Random-bred CD1 mice were purchased from Charles River Laboratories. Mice were paired overnight, and females were checked for copulation plugs the next morning, designated day E0.5. Null mutant embryos for *Casp3* and *Apaf1* and corresponding wild-type embryos ($n = 3$ for each genotype) were obtained and genotyped as reported previously (13, 30).

Whole Embryo Culture. Embryos were explanted and cultured in rat serum as described previously (15). Yolk sac circulation, crown-rump length, and somite number provided measures of viability, growth, and developmental progression, respectively (31). Stock solutions of z-VAD-fmk (Sigma-Aldrich) in DMSO and pifithrin-α (Calbiochem) in PBS were added to cultures as 0.1% (vol/vol) additions to obtain the final concentrations of 200 μM and 500 μM, respectively. Initial experiments demonstrated that these doses were not embryotoxic at this developmental stage. An equivalent volume of DMSO was added to control cultures, because no difference between PBS and DMSO (at 0.1%) was observed in initial experiments. Embryos were randomly allocated to treatment groups to minimize the effect of litter-to-litter variation. Embryos were rinsed in PBS and fixed in 4% paraformaldehyde for immunohistochemistry, in situ hybridization, or TUNEL analysis.

Immunohistochemistry. Embryos were embedded in paraffin wax, and 7-μm transverse sections were processed for immunostaining using antibodies specific for phospho-histone H3 (Upstate Biotechnology), activated caspase-3 (Cell Signaling Technology), and E-cadherin (Cell Signaling Technology). Sections were counterstained with methyl green. The numbers of positive cells were counted as described previously (32, 33).

Whole-Mount In Situ Hybridization. A digoxygenin-labeled cRNA probe for *Cadherin-6* was used as described previously (34).

TUNEL of Whole Embryos. Embryos were fixed in 4% paraformaldehyde, and whole-mount TUNEL was performed as described previously (35).

Statistical Analysis. Statistical tests were performed using SigmaStat version 3.5 (Systat Software).

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1. Copp AJ, Greene NDE, Murdoch JN (2003) The genetic basis of mammalian neurulation. *Nat Rev Genet* 4:784–793.
2. Copp AJ (2005) Neurulation in the cranial region: Normal and abnormal. *J Anat* 207:623–635.
3. Harris MJ, Juriloff DM (2007) Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. *Birth Defects Res A Clin Mol Teratol* 79:187–210.
4. Ybot-Gonzalez P, et al. (2007) Convergent extension, planar-cell-polarity signaling and initiation of mouse neural tube closure. *Development* 134:789–799.
5. Ybot-Gonzalez P, et al. (2007) Neural plate morphogenesis during mouse neurulation is regulated by antagonism of BMP signaling. *Development* 134:3203–3211.
6. Schluter G (1973) Ultrastructural observations on cell necrosis during formation of the neural tube in mouse embryos. *Z Anat Entwickl-Gesch* 141:251–264.
7. Geelen JAG, Langman J (1979) Ultrastructural observations on closure of the neural tube in the mouse. *Anat Embryol* 156:73–88.
8. Mirkes PE (2002) 2001 Warkany Lecture. To die or not to die: The role of apoptosis in normal and abnormal mammalian development. *Teratology* 65:228–239.
9. Weil M, Jacobson MD, Raff MC (1997) Is programmed cell death required for neural tube closure? *Curr Biol* 7:281–284.
10. Mirkes PE, Little SA (1998) Teratogen-induced cell death in postimplantation mouse embryos: Differential tissue sensitivity and hallmarks of apoptosis. *Cell Death Differ* 5:592–600.
11. Mirkes PE, Little SA, Umpierre CC (2001) Co-localization of active caspase-3 and DNA fragmentation (TUNEL) in normal and hyperthermia-induced abnormal mouse development. *Teratology* 63:134–143.
12. Kumar S (2007) Caspase function in programmed cell death. *Cell Death Differ* 14:32–43.
13. Leonard JR, Klocke BJ, D'Sa C, Flavell RA, Roth KA (2002) Strain-dependent neurodevelopmental abnormalities in caspase-3–deficient mice. *J Neuropathol Exp Neurol* 61:673–677.
14. Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA, Gruss P (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* 94:727–737.
15. Cockroft DL (1990) in *Postimplantation Mammalian Embryos*: *A Practical Approach*, eds Copp AJ, Cockroft DL (IRL, Oxford), pp. 15–40.
16. Murphy PJ, et al. (2004) Pifithrin-alpha inhibits p53 signaling after interaction of the tumor suppressor protein with hsp90 and its nuclear translocation. *J Biol Chem* 279:30195–30201.
17. Van Straaten HWM, Hekking JWM, Copp AJ, Bernfield M (1992) Deceleration and acceleration in the rate of posterior neuropore closure during neurulation in the curly tail (ct) mouse embryo. *Anat Embryol* 185:169–174.
18. Homanics GE, et al. (1995) Exencephaly and hydrocephaly in mice with targeted modification of the apolipoprotein B (*Apob*) gene. *Teratology* 51:1–10.
19. Ruland J, et al. (2001) Bcl10 is a positive regulator of antigen receptor–induced activation of NF-kappaB and neural tube closure. *Cell* 104:33–42.
20. Migliorini D, et al. (2002) Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol Cell Biol* 22:5527–5538.
21. Ikeda A, Ikeda S, Gridley T, Nishina PM, Naggert JK (2001) Neural tube defects and neuroepithelial cell death in *Tulp3* knockout mice. *Hum Mol Genet* 10:1325–1334.
22. Sah VP, et al. (1995) A subset of *p53*-deficient embryos exhibit exencephaly. *Nat Genet* 10:175–180.
23. Kuida K, et al. (1998) Reduced apoptosis and cytochrome c–mediated caspase activation in mice lacking caspase 9. *Cell* 94:325–337.
24. Baehrecke EH (2002) How death shapes life during development. *Nat Rev Mol Cell Biol* 3:779–787.
25. Sugishita Y, Leifer DW, Agani F, Watanabe M, Fisher SA (2004) Hypoxia-responsive signaling regulates the apoptosis-dependent remodeling of the embryonic avian cardiac outflow tract. *Dev Biol* 273:285–296.
26. Cuervo R, Valencia C, Chandraratna RA, Covarrubias L (2002) Programmed cell death is required for palate shelf fusion and is regulated by retinoic acid. *Dev Biol* 245:145–156.
27. Takahara S, Takigawa T, Shiota K (2004) Programmed cell death is not a necessary prerequisite for fusion of the fetal mouse palate. *Int J Dev Biol* 48:39–46.
28. Shum ASW, Copp AJ (1996) Regional differences in morphogenesis of the neuroepithelium suggest multiple mechanisms of spinal neurulation in the mouse. *Anat Embryol* 194:65–73.
29. Selcuki M, Vatansever S, Umur AS, Temiz C, Sayin M (2008) Apoptosis seems to be the major process while surface and neural ectodermal layers detach during neurulation. *Childs Nerv Syst* 24:577–580.
30. Kuida K, et al. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384:368–372.
31. Dunlevy LPE, Burren KA, Chitty LS, Copp AJ, Greene NDE (2006) Excess methionine suppresses the methylation cycle and inhibits neural tube closure in mouse embryos. *FEBS Lett* 580:2803–2807.
32. Dunlevy LPE, et al. (2006) Integrity of the methylation cycle is essential for mammalian neural tube closure. *Birth Defects Res A Clin Mol Teratol* 76:544–552.
33. Gustavsson P, et al. (2007) Increased expression of Grainyhead-like-3 rescues spina bifida in a folate-resistant mouse model. *Hum Mol Genet* 16:2640–2646.
34. Henderson DJ, Ybot-Gonzalez P, Copp AJ (1997) Over-expression of the chondroitin sulphate proteoglycan *versican* is associated with defective neural crest migration in the *Pax3* mutant mouse (*splotch*). *Mech Dev* 69:39–51.
35. Martinez-Barbera JP, et al. (2002) Folic acid prevents exencephaly in *Cited2*-deficient mice. *Hum Mol Genet* 11:283–293.