SPECIAL ISSUE RESEARCH ARTICLE

Xenopus hairy2 Functions in Neural Crest Formation by Maintaining Cells in a Mitotic and Undifferentiated State

Kan-Ichiro Nagatomo and Chikara Hashimoto*

The neural crest is a population of mitotically active, multipotent progenitor cells that arise at the neural plate border. Neural crest progenitors must be maintained in a multipotent state until after neural tube closure. However, the molecular underpinnings of this process have yet to be fully elucidated. Here we show that the basic helix-loop-helix (bHLH) transcriptional repressor gene, *Xenopus hairy2* (*Xhairy2*), is an essential early regulator of neural crest formation in *Xenopus*. During gastrulation, *Xhairy2* is localized at the presumptive neural crest prior to the expression of such neural crest markers as *Slug* and *FoxD3*. Morpholino-mediated knockdown of Xhairy2 results in the repression of neural crest marker gene expression while inducing the ectopic expression of the cell cycle inhibitor $p27^{xic1}$ in the presumptive neural crest. We also found that ectopic $p27^{xic1}$ disturbs neural crest formation. Furthermore, the depletion of Xhairy2 leads to the apoptosis of mitotic cells. Our results suggest that *Xhairy2* functions in neural crest specification by maintaining cells in the mitotic and undifferentiated state. *Developmental Dynamics 236: 1475–1483, 2007.* © 2007 Wiley-Liss, Inc.

Key words: neural crest; *Xhairy2*; *Foxd3*; *Slug*; $p27^{xic1}$; *X-Delta-1*

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INTRODUCTION

The neural crest is a multipotent and transient population of ectoderm derivatives that arise from the border between the neural plate and epidermis in vertebrate embryos. As the neural tube closes, neural crest cells delaminate and migrate to diverse locations. They differentiate into diverse cell types that include neurons and glia of the peripheral nervous system, smooth muscle cells, craniofacial cartilage, pigment cells, bone, and fin (LaBonne and Bronner-Fraser, 1999; Christiansen et al., 2000).

Many studies have focused on the induction of neural crest precursors. Some extracellular signaling molecules such as BMP, Wnt, FGF, and Notch are believed to up-regulate specific target transcription factors that have been shown to be required for neural crest formation (LaBonne and Bronner-Fraser, 1998; Huang and Saint-Jeannet, 2004; Steventon et al., 2005; Cornell and Eisen, 2005). The combinatorial action of signaling molecules and these transcription factors defines the bona fide neural crest region (reviewed by Sauka-Spengler and Bronner-Fraser, 2006), although little is known about how these signaling molecules and transcription factors regulate neural crest induction and maintenance. Concomitant with the induction of neural crest precursors, the neural crest precursors must be multipotent progenitors. It has been suggested that there should be some protective mechanism that prevents neural crest precursors from adopting these alternate fates and immature differentiation, so that the neural crest precursors can be generated adjacent to signals that influence both neural plate and prospective epidermal cell fates. For example, the proto-oncogenic protein c-Myc and its direct target gene ID3 were suggested to play an essential role in maintaining neural crest progenitors in the multipotent state (Bellmeyer et al., 2003; Light et al., 2005; Kee and Bronner-Fraser, 2005), yet little is known about the molecular underpinnings of this process.

Hairy and enhancer of split (hes)-

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related genes are known to function in the establishment of various tissue identities in both vertebrates and invertebrates (Fisher and Caudy, 1998; Davis and Turner, 2001). In particular, hes-related genes have a prominent role in inhibiting neurogenesis. Recent studies have revealed that the timing of neural stem cell differentiation is critically controlled by multiple hes-related genes (reviewed by Kageyama et al., 2005). Additionally, in general, differentiation is closely related to the cell cycle, and hes-related genes also act to control cell cycle progression. Hes1 directly contributes to the promotion of progenitor cell proliferation through transcriptional repression of a cyclin-dependent kinase inhibitor, p27Kip1, in embryonic carcinoma cells (Murata et al., 2005). Zebrafish *p27^{xic1}* expression is negatively regulated by Her5 in the midbrain-hindbrain boundary (Geling et al., 2003). These findings suggested that hes-related genes might be involved in maintaining neural crest stem cells in the mitotic and undifferentiated state.

In *Xenopus*, a member of the hes-related gene, *Xhairy2*, is expressed in the entire prospective ectodermal region prior to the gastrula stage. As gastrulation progresses, the ectodermal expression of *Xhairy2* is localized as a narrow stripe that curves and forms a border between the neural plate and the epidermis during gastrulation (Tsuji et al., 2003). Although *Xhairy2* has been suggested to be the transcriptional effector of Notch signaling and to act as a repressor of *Xbmp4* transcription during cranial neural crest induction (Glavic et al., 2004), its role in neural crest development is largely unknown. Here, we report that the morpholino-mediated knockdown of Xhairy2 results in the repression of neural crest marker gene expression and neural crest derivatives. Our results also suggest that the depletion of Xhairy2 affects neural crest cell proliferation and survival, and the neural crest derivatives, in turn, are abolished. Furthermore, our data indicate that *Xhairy2* is essential for the repression of cdk inhibitor *p27^{xic1}* expression in the presumptive neural crest region from the gastrula stage, and this repression is likely to be responsible for neural crest specification. We propose that *Xhairy2* functions in early neural crest specification by maintaining cells in the mitotic and undifferentiated state.

RESULTS

Xhairy2 Is Required for Neural Crest Development

Xhairy2 expression is localized at the neural plate border that contributes to the neural crest at the early neurula stage (Tsuji et al., 2003; see also Fig. 1A), and the overexpression of *Xhairy2* results in an increase of the neural crest population (Glavic et al., 2004). We, therefore, examined the roles of *Xhairy2* in the normal development of the presumptive neural crest region by performing loss-of-function studies using morpholino antisense oligonucleotides (MOs). Injection of Xhairy2-MO led to repression of the expression of such neural crest markers as *Slug* and *FoxD3* at the early neurula stage (*Slug*, 86%, n = 72; *FoxD3*, 90%, n = 82; Fig. 1C,F), whereas injection of Control-MO did not affect neural crest marker expression (*Slug*, 100% normal, n = 31; *FoxD3*, 100% normal, n = 42; Fig. 1B,E). This disappearance of the neural crest markers was rescued by coinjection of *Xhairy2* mRNA that does not contain the morpholino annealing site (*Slug*, 29% inhibition, n = 76; *FoxD3*, 30% inhibition, n = 64; Fig. 1D,G). These results suggest that *Xhairy2* is required for the early development of the neural crest.

The loss of early neural crest marker expression in Xhairy2-depleted embryos suggests that *Xhairy2* may play important role(s) in neural crest development. To this end, we next attempted to examine what happens to neural crest derivatives in Xhairy2-depleted embryos at later stages. Embryos injected with Xhairy2-MO showed repression of both *Slug* and *FoxD3* expressions at mid neurula stage (*Slug*, 72%, n = 25; *FoxD3*, 66%, n = 29; Fig. 2C,D), whereas injection of Control-MO did not affect neural crest marker expression (*Slug*, 100% normal, n = 24; *FoxD3*, 100% normal, n = 25; Fig. 2A,B). Embryos injected with Xhairy2-MO showed reduced number of migratory neural crest cells as depicted by *Xtwist* expression at stage 24 (68%, n = 28; Fig. 2F). In Xhairy2-depleted embryos at stage 25, we detected no *n-tubulin*-positive cranial nerves, which are known to be the derivatives of cranial neural crest (64%, n = 22; Fig. 2H). At stage 45, the branchial arch cartilage and other derivative tissues of the cranial neural crest almost completely disappeared (50%, n = 6; Fig. 2K) on the injected side. The mesenchymes of dorsal fin and melanocytes originating in the trunk neural crest were not formed in embryos injected with Xhairy2-MO (56%, n = 16; Fig. 2M). Therefore, the loss of neural crest derivatives in Xhairy2-depleted embryos appears to be a consequence of early hypoplasia of the neural crest region.

Xhairy2 Affects Neural Crest Development in a Cell-Autonomous Manner

As shown above, *Xhairy2* seems to be involved in the development of the neural crest from an early stage. So how does *Xhairy2* function in this process? It has been surmised that an interaction between the neural plate and the epidermis is involved in the induction of the neural crest (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996). Thus, it may be possible that *Xhairy2* affects the formation of either the neural plate or the epidermis, and the development of the neural crest, in turn, is affected indirectly. However, the expression patterns of both neural plate markers (*Xsox2* and *Xsox3*) and the epidermal marker (*Keratin*) were observed in Xhairy2-depleted embryos (*Xsox2*, 100%, n = 56; *Keratin*, 100%, n = 53; Fig. 1H,I, and data not shown), suggesting that the loss of early neural crest marker expression by Xhairy2-MO may have a direct effect on the neural crest.

Another possibility is that *Xhairy2* may regulate the expression of *Xbmp4*, since it was reported that Notch signaling might participate in neural crest specification by down-regulating *Xbmp4* transcription through the activation of *Xhairy2A* (Glavic et al., 2004). However, the depletion of Xhairy2 did not lead to any changes in the expression patterns of *Xbmp4* from early gastrula to early neurula stage (100%, n = 46; Fig. 1J,K

and data not shown). We also found that the overexpression of *X-Delta-1*^{stu}, which inhibits Notch signaling (Chitnis et al., 1995), up-regulates *Xbmp4* expression (data not shown), in agreement with a previous study (Kuriyama et al., 2006), suggesting that the up-regulation of *Xbmp4* expression by Notch signaling inhibition is mediated by not only *Xhairy2* but also other Notch signaling target molecules.

Taken together, we conclude that *Xhairy2* cell-autonomously regulates the expression of early neural crest markers, completely independent of the formation of other tissues.

Depletion of Xhairy2 Does Not Lead to Premature Neuronal Differentiation, But Induces Ectopic *p27*^{xic1} and *X-Delta-1* Expression in Presumptive Neural Crest

Because previous studies have indicated that *Xhairy2* is an antineurogenic gene (Dawson et al., 1995; Stancheva et al., 2003) and Notch signaling promotes formation of neural crest by repressing neurogenin1 function (Cornell and Eisen, 2002), the depletion of Xhairy2 may lead to premature neuronal differentiation that could cause the loss of early neural crest markers. To examine this possibility, we analyzed the expression of the neural differentiation marker *n-tubulin* (Chitnis et al., 1995) and the neuronal determination gene *X-ngnr-1* (Ma et al., 1996) in Xhairy2-depleted embryos. Xhairy2-MO injection led to a slight increase of *n-tubulin* and *X-ngnr-1* expression around trigeminal ganglia (*n-tubulin*, 39%, n = 31; *X-ngnr-1*, 38%, n = 26; Fig. 3A,B), but

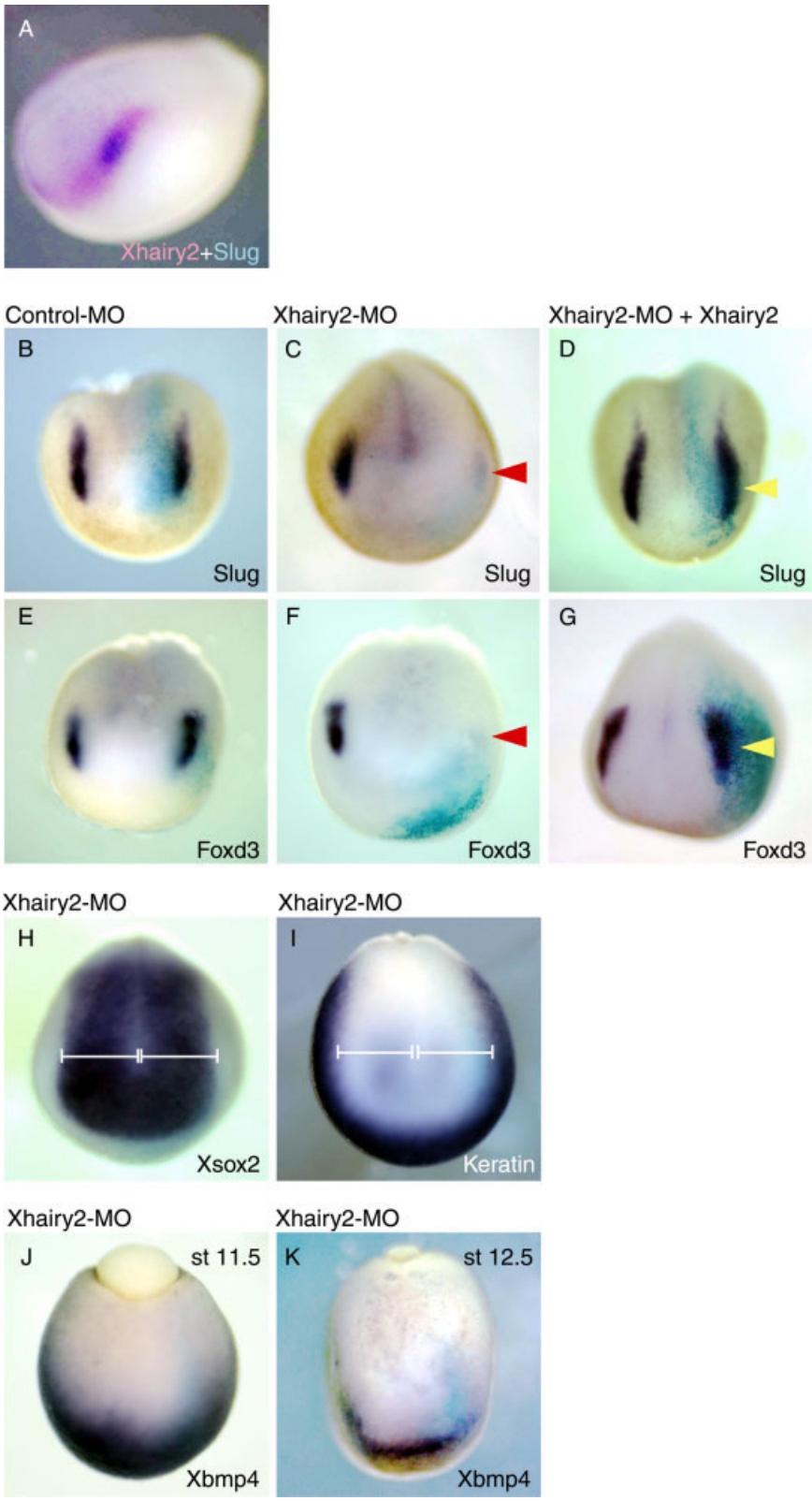

Fig. 1. *Xhairy2* is required for neural crest marker gene expression. **A:** Double in situ hybridization with *Xhairy2* (magenta) and *Slug* (blue) at stage 13. **B,E:** Control-MO injection has no effect on any of the neural crest markers examined at stage 13: *Slug* (B) and *FoxD3* (E). **C,F:** In embryos injected with Xhairy2-MO, the expression of *Slug* (C) and *FoxD3* (F) is inhibited on the injected side (red arrowhead) at stage 13. **D,G:** Loss of neural crest marker expression in embryos injected wit Xhairy2-MO is rescued by injecting 20 pg of *Xhairy2* mRNA (yellow arrowhead): *Slug* (D) and *FoxD3* (G). **H,I:** In embryos injected with Xhairy2-MO, the expression of *Xsox2* (H) and *Keratin* (I) is not apparently affected at stage 13. White brackets indicate the width of the neural plate. **J,K:** In embryos injected with Xhairy2-MO, *Xbmp4* expression is not affected at stages 11.5 (J) and 12.5 (K). A, lateral views with anterior to the left. B–K, dorsal views with anterior to the bottom. Injected area is the right side. Turquoise staining is lineage tracer β-gal.

not in the neural crest. These results indicate that the early repression of neural crest marker expression by Xhairy2-MO may not depend upon the premature neuronal differentiation.

We also examined the expression of the neurogenic gene *X-Delta-1* (Chitnis et al., 1995) and the cell cycle inhibitor $p27^{xic1}$ that is required for primary neurogenesis (Vernon et al., 2003) in the absence of Xhairy2. We found that the expression patterns of both $p27^{xic1}$ and *X-Delta-1* were affected, and that those ectopic expressions were repressed by co-injecting *Xhairy2* mRNA, suggesting that those effects are specific to *Xhairy2*. As regards the expression pattern of $p27^{xic1}$, broad ectopic expression was observed in the ectoderm including the presumptive neural crest (96%, n = 84; Fig. 3D), and this ectopic expression was completely repressed by co-injecting *Xhairy2* mRNA (91% n = 46; Fig. 3E). This up-regulation was observed from the early gastrula stage (data not shown). In good agreement with this result, *Xhairy2* expression is located in the anterior region and $p27^{xic1}$ expression is in the posterior region, appearing to complement each other from the early gastrula stage (Fig. 3I,J and data not shown). In addition, we noted the weak ectopic expression of *X-Delta-1* in the cranial neural crest region (75%, n = 40; Fig. 3G), and this ectopic expression was completely repressed by co-injecting *Xhairy2* mRNA (100% n = 33; Fig. 3H). A previous study showed that *Slug* expression is surrounded by *X-Delta-1* expression and they never overlap in early neurula embryo (Glavic et al., 2004). Together, these data indicate that $p27^{xic1}$ and/or *X-Delta-1* may hinder cells from taking the neural crest fate, and *Xhairy2* represses the expression of these genes to establish neural crest identity.

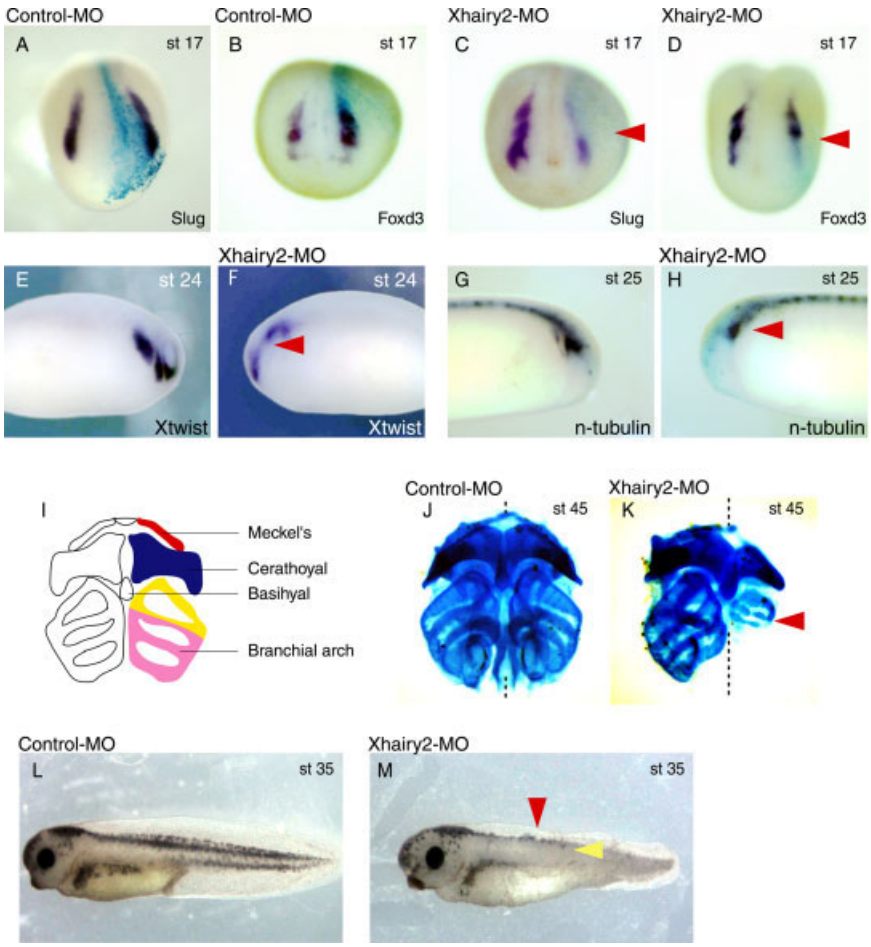

Fig. 2. Depletion of Xhairy2 leads to loss of neural crest derivatives. **A,B:** Control-MO injection has no effect on any of the neural crest markers examined at stage 17: *Slug* (A) and *FoxD3* (B). **C,D:** In embryos injected with Xhairy2-MO, the expression of *Slug* (C) and *FoxD3* (D) in inhibited on the injected side (red arrowhead) at stage 17. **E,F:** Compared with the control side (E), the Xhairy2-MO injected side shows reduced migratory neural crest cells (F) as depicted by *Xtwist* expression (red arrowhead) at stage 24. **G,H:** Compared with the control side (G), the Xhairy2-depleted embryo shows no cranial sensory ganglia on the injected side (H) as depicted by *n-tubulin* expression (red arrowhead) at stage 25. **I:** Schematic diagram depicting ventral cranial cartilages of a stage 45 embryo. **J:** Cartilage staining of Control-MO injected embryo shows normal structures at stage 45. **K:** Cartilage staining of Xhairy2-MO injected embryo shows partial loss of cartilage on the injected side (red arrowhead) at stage 45. **L,M:** Phenotypes of stage 35 embryos whose bilateral trunk neural crest was injected with Control-MO or Xhairy2-MO. Melanocytes of Xhairy2-MO injected embryo are reduced (M; yellow arrowhead) compared with Control-MO injected embryo (L). L: The dorsal fin of Control-MO injected embryo shows normal structures M: The dorsal fin of Xhairy2-MO injected embryo shows partial loss (red arrowhead). A–D, dorsal views with anterior to the bottom. E,G, lateral views with anterior to the right. F, H, L, M, lateral views with anterior to the left. J, K, ventral views with anterior to the top. A–D,J,K, injected area is the right side. Turquoise staining is lineage tracer β-gal.

Repression of $p27^{xic1}$ Expression by *Xhairy2* Is Involved in Early Neural Crest Specification

During gastrulation, *Xhairy2* represses $p27^{xic1}$ expression in the ectoderm that includes the presumptive neural crest prior to the expression of early neural crest markers. To examine whether the repression of $p27^{xic1}$ expression by *Xhairy2* is required for the expression of early neural crest markers, we first overexpressed $p27^{xic1}$ mRNA. The overexpression of $p27^{xic1}$ inhibited *Slug* and *FoxD3* expression (*Slug*, 83%, n = 36; *FoxD3*, 73%, n = 40; Fig. 4A,B). These effects are similar to that of Xhairy2-MO. *X-Delta-1* expression was slightly increased by $p27^{xic1}$ overexpression immediately outside the cranial neural crest region (52%, n = 44; Fig. 4C). Therefore, the repression of *Slug* and *FoxD3* expression by Xhairy2-MO could be realized, at least in part, by the ectopic expression of $p27^{xic1}$.

To further evaluate the effect of $p27^{xic1}$ repression by *Xhairy2* on early neural crest development, we next examined whether early changes of neural crest marker and *X-Delta-1* expression by Xhairy2-MO are rescued by $p27^{xic1}$-MO. The injection of $p27^{xic1}$-MO resulted in a slightly increased number

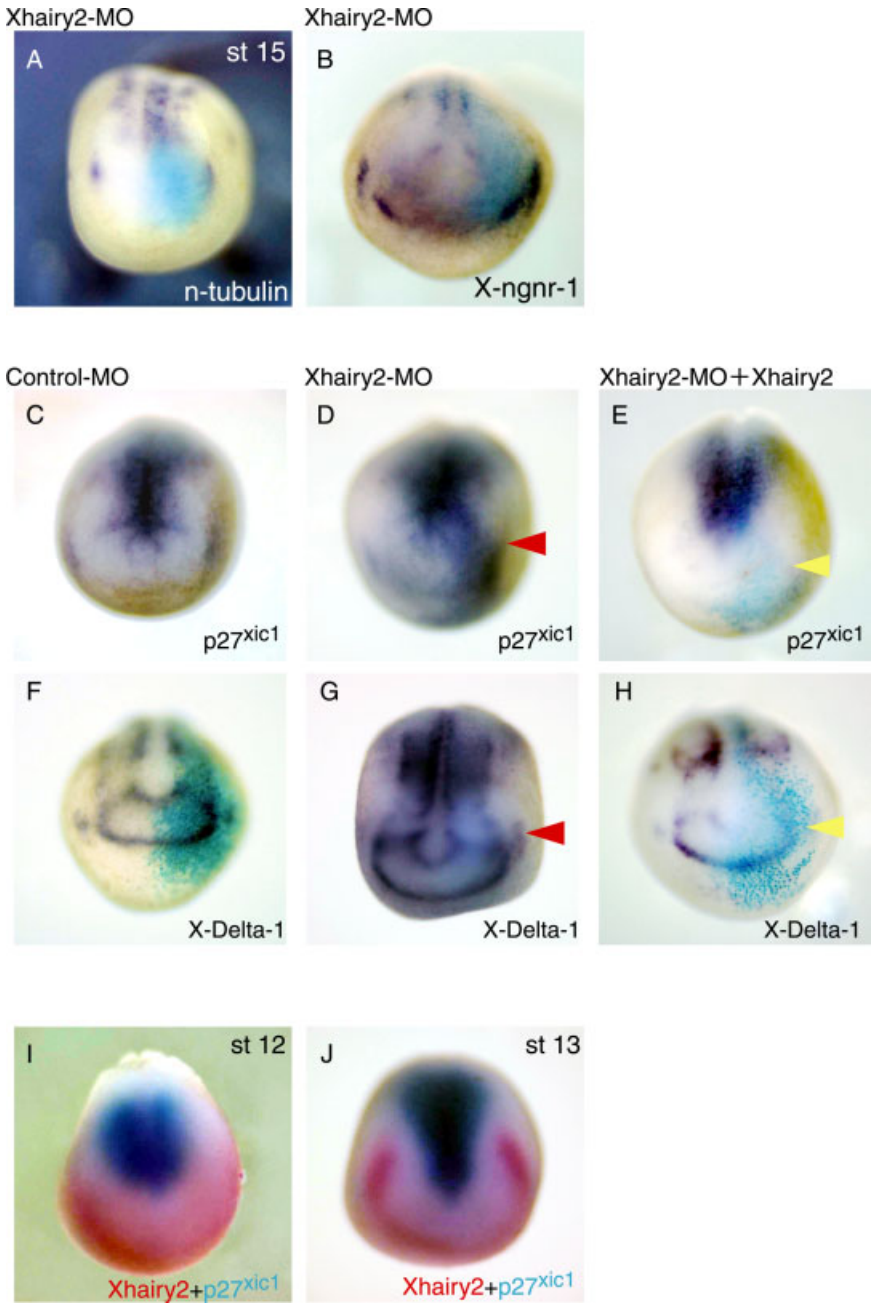

Fig. 3. Depletion of Xhairy2 induces ectopic *p27^{xic1}* and *X-Delta-1* expression in presumptive neural crest. **A,B:** Xhairy2-MO induces slight increases of *n-tubulin* expression at stage 15 (A) and *X-ngnr-1* expression at stage 13 (B) in trigeminal ganglia. **C:** Control-MO injection has no effect on *p27^{xic1}* expression at stage 13. **D:** In embryos injected with Xhairy2-MO, ectopic expression of *p27^{xic1}* is induced on the injected side (red arrowhead) at stage 13. **E:** The ectopic expression of *p27^{xic1}* is repressed by injecting 20 pg of *Xhairy2* mRNA (yellow arrowhead). **F:** Control-MO injection has no effect on *X-Delta-1* expression at stage 13. **G:** Xhairy2-MO induces a modest increase of *X-Delta-1* expression in the cranial neural crest region (red arrowhead) at stage 13. **H:** The modest increase of *X-Delta-1* expression is repressed by injecting 20 pg of *Xhairy2* mRNA (yellow arrowhead). **I:** Double in situ hybridization with *Xhairy2* (blue) and *p27^{xic1}* (magenta) at stage 12. **J:** Double in situ hybridization with *Xhairy2* (blue) and *p27^{xic1}* (magenta) at stage 13. Dorsal views with anterior to the bottom. Injected area is the right side. Turquoise staining is lineage tracer β-gal.

of apoptotic cells, but did not significantly affect neural crest marker or *X-Delta-1* expression (*Slug*, 100% normal, n = 24; *FoxD3*, 100% normal, n = 28; *X-Delta-1*, 100% normal, n = 20; Fig. 4D–F and data not shown). The repression of early neural crest marker expression by Xhairy2-MO was partially rescued by co-injecting p27^{xic1}-MO with Xhairy2-MO (*Slug*, 48% inhibition, n = 69; *FoxD3*, 51% inhibition, n = 67; Fig. 4J,K), but not by co-injecting Control-MO (*Slug*, 75% inhibition, n = 56; *FoxD3*, 83% inhibition, n = 64; Fig. 4G,H). Similar results were observed for *X-Delta-1* expression in the cranial neural crest region (Control-MO + Xhairy2-MO, 84% ectopic expression, n = 61; p27^{xic1}-MO + Xhairy2-MO, 54% ectopic expression, n = 72; Fig. 4I,L). The finding that the percentage of rescued early neural crest marker and *X-Delta-1* expression by p27^{xic1}-MO is lower than that by *Xhairy2* mRNA, indicates that the effect of Xhairy2-MO on early neural crest marker and *X-Delta-1* expression is due not only to the ectopic *p27^{xic1}* expression. This is supported by the fact that the overexpression of *p27^{xic1}* did not induce the ectopic expression of *X-Delta-1* in the cranial neural crest region.

Taken together, these results suggest that the repression of *p27^{xic1}* expression by *Xhairy2* plays a role in early neural crest marker expression and the repression of *X-Delta-1* expression in the presumptive neural crest region.

Xhairy2 Is Required for Cell Proliferation and Survival in Neural Crest

As shown above, *Xhairy2* represses the expression of the cell cycle inhibitor *p27^{xic1}*. Thus, *Xhairy2* seems to be required for controlling the cell cycle in the neural crest. To check this possibility, we performed both immunostaining with anti-phosphohistone H3 and TUNEL staining of Xhairy2-depleted embryos, and found a decrease in the number of proliferating cells (42%, n = 12; Fig. 5D) as well as significantly increased apoptosis in mid neurula stage embryos injected with Xhairy2-MO (92%, n = 25; Fig. 5H), whereas injection of Control-MO did not affect cell cycle regulation (100%, normal number of proliferating cells, n = 15; Fig. 5C; 100% normal number of apoptotic cells, n = 20; Fig. 5G). Interestingly, these effects on cell cycle regulation were not observed from early gastrula through the early neurula stage (100%, normal number of proliferating cells, n = 31; Fig. 5B and data not shown; 100% normal number

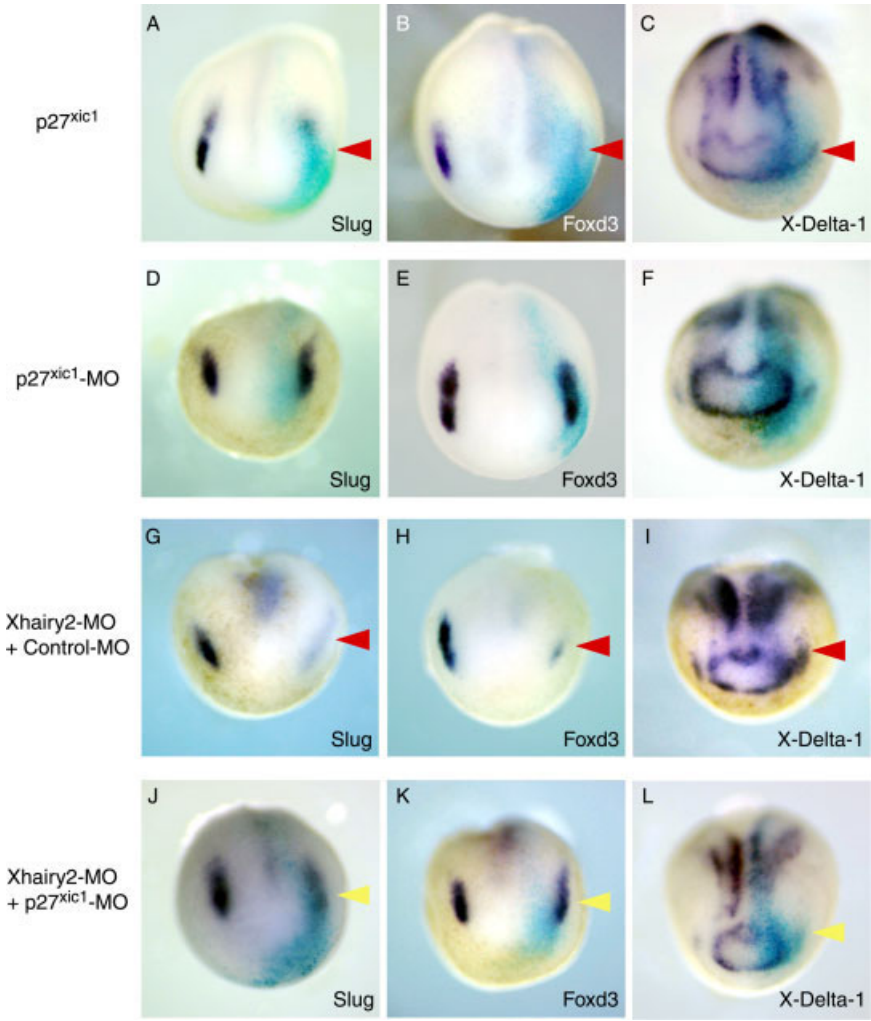

Fig. 4.

of apoptotic cells, n = 53; Fig. 5F and data not shown). How can these differences between early and mid neurula stages be explained? There are at least two interpretations of these results. One is that *p27^{xic1}* protein levels might not be sufficient for cell cycle control at the early neurula stage. The other interpretation is that decreased cell proliferation and increased apoptosis might be a direct consequence of not only ectopic *p27^{xic1}* expression but also combined effects, such as the repression of some neural crest markers that are known to control cell proliferation and apoptosis.

Taken together, the results suggest that *Xhairy2* is essential for neural crest cell proliferation and survival.

DISCUSSION

In this report, we show that the specification of neural crest cells is dependent in part upon the function of *Xhairy2*, indicating the existence of novel mechanism(s) for neural crest specification closely associated with *Xhairy2* function.

It is known that a high BMP concentration induces the epidermis, and a significantly low BMP concentration induces the neural plate (reviewed by Sasai and De Robertis, 1997). Since BMP is a secreted molecule, an intermediate concentration should be automatically generated at the border between neural and epidermal tissues. It is known that an intermediate concentration of BMP is needed for neu-

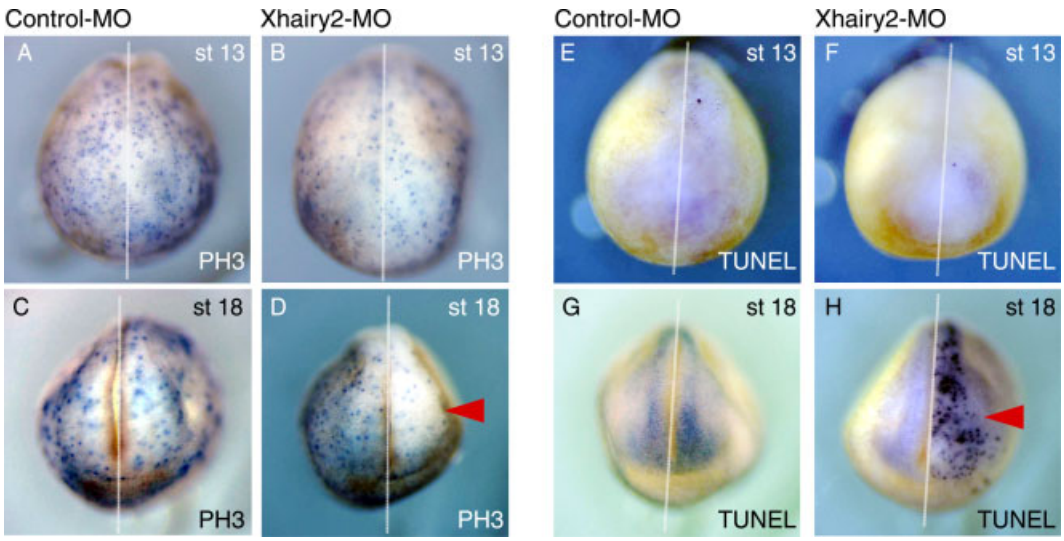

Fig. 5.

ral crest specification (Marchant et al., 1998). Although *Xhairy2* in the prospective neural crest is known to down-regulate *Xbmp4* transcription to the suitable concentration for neural crest induction as a Notch signaling target gene (Glavic et al., 2004), our results showed that the ablation of Xhairy2 had no effect on the expression of *Xbmp4* (Fig. 1J,K). Instead, the ectopic expression of a dominant-negative form of *XBMPRII* (Frisch and Wright, 1998) or *BMP3b*, which partially blocks BMP signaling (Hino et al., 2003), up-regulates *Xhairy2* expression ectopically (data not shown). In agreement with a previous study by Kuriyama et al. (2006), our results indicate that an intermediate concentration of BMP is necessary for the induction of *Xhairy2* expression at the neural border.

The primary induction of ectoderm into epidermis, neural crest, or neural plate occurs at the early gastrula stage. *Xhairy2* is expressed initially in the entire ectoderm prior to gastrulation, and then its expression is restricted to a narrow band at the border between the neural plate and the epidermis (Tsuji et al., 2003). This means that *Xhairy2* is down-regulated in both neural and epidermal cells, or the gene expression continues to be maintained only in prospective neural crest cells. It is known that epidermal cells are specified at high BMP concentration and that the neural plate is formed in the absence of BMP. Thus, only cells receiving intermediate BMP signals would incompletely differentiate into either tissue, and only those cells would retain *Xhairy2* expression.

Neural crest cells are pluripotent (reviewed by LaBonne and Bronner-Fraser, 1999). Considering this property, prospective neural crest cells should keep their proliferative ability until the time of differentiation. Cyclin-dependent kinases (CDKs) positively regulate the cell cycle, and $p27^{xic1}$ inhibits CDKs involved in the G1/S transition (Sherr and Roberts, 1999). This cell cycle regulation is thought to be important for the transition between differentiation and proliferation. Indeed, $p27^{xic1}$ is highly expressed in cells destined to become primary neurons, and it is required for primary neurogenesis (Vernon et al., 2003). Previous studies have shown that the timing of neural stem cell differentiation is critically controlled by multiple hes-related genes (reviewed by Kageyama et al., 2005). Generally, differentiation is closely related to the cell cycle and hes-related gene products control cell cycle progression. For example, Hes1 directly contributes to the promotion of progenitor cell proliferation through transcriptional repression of the cdk inhibitor $p27^{kip1}$ in embryonic carcinoma cells (Murata et al., 2005). Thus, it is suggested that once $p27^{xic1}$ is expressed, the cells are going to assume certain cell fates by escaping from the proliferation phase. As shown above, the ablation of Xhairy2 leads to the inhibition of neural crest formation (Figs. 1, 2), and this inhibition is partially dependent upon the ectopic expression of $p27^{xic1}$ (Figs. 3, 4). Thus, *Xhairy2* may function to maintain prospective neural crest cells in the mitotic and undifferentiated state. Supporting this idea, the ablation of Xhairy2 induced the apoptosis of mitotic cells (Fig. 5), and $p27^{xic1}$-MO reduced the increased population of apoptotic cells in the Xhairy2 ablated embryos (data not shown). $p27^{xic1}$-MO, however, did not rescue the reduction of the number of mitotic cells caused by Xhairy2-MO (data not shown). As shown above, $p27^{xic1}$-MO does not completely rescue the phenotype of Xhairy2 knockdown. Therefore, it is possible to think that *Xhairy2* may have another function(s), which positively progresses the cell cycle in addition to the repression of the expression of the cell cycle inhibitor, $p27^{xic1}$.

We showed that the ablation of Xhairy2 leads to the ectopic expression of $p27^{xic1}$, and the ectopically expressed $p27^{xic1}$, in turn, suppresses neural crest formation. However, $p27^{xic1}$ does not seem to directly regulate the gene expression by itself. In addition, the ectopic expression of *Xhairy2* does not cause the ectopic induction of gene expressions specific for the neural crest (Glavic et al., 2004; data not shown), indicating that the gene expression should be directly regulated by other mechanism(s). Several recent reports have indicated that Wnts are neural crest inducers (reviewed by Raible, 2006). It was reported that the cooperative function of *Pax3* and *Zic1*, in concert with Wnt signaling, is essential for neural crest specification (Monsoro-Burq et al., 2005; Sato et al., 2005). The transcription factor *Xsnail*, which is expressed as early as *Xhairy2* in the prospective neural crest, is known to have the ability to induce neural crest marker genes (Aybar et al., 2003). Therefore, the gene regulatory network that includes these genes may be involved in the direct induction of the neural

Fig. 4. Repression of $p27^{xic1}$ expression by *Xhairy2* is involved in early neural crest specification. **A,B:** Embryo injected with 10 pg of $p27^{xic1}$ mRNA shows repression of *Slug* (A) and *Foxd3* (B) expression (red arrowhead). **C:** The injection of 10 pg of $p27^{xic1}$ mRNA produces a slightly increase of *X-Delta-1* expression on the lateral side of the neural plate (red arrowhead). **D–F:** $p27^{xic1}$-MO injection has no effect on *X-Delta-1* expression (F) or any of the neural crest markers examined: *Slug* (D) and *Foxd3* (E). **G,H,J,K:** The repression of early neural crest marker expression by Xhairy2-MO seems to be rescued by $p27^{xic1}$-MO injection (yellow arrowhead): *Slug* (J) and *Foxd3* (K), but not by Control-MO injection (red arrowhead): *Slug* (G) and *Foxd3* (H). **I,L:** The mild increase of *X-Delta-1* expression by Xhairy2-MO seems to be rescued by $p27^{xic1}$-MO injection (L) but not by Control-MO injection (I). Dorsal views with anterior to the bottom at stage 13. Injected area is the right side. Turquoise staining is lineage tracer β-gal.

Fig. 5. *Xhairy2* is required for cell proliferation and survival in neural crest. **A–D:** Phosphohistone H3 immunohistochemistry of Xhairy2-MO or Control-MO injected embryos. A,C: There is no detectable difference in the number of actively cycling cells between the side injected with Control-MO and the uninjected side at stages 13 (A) and 18 (C). B: There is no detectable difference in the number of actively cycling cells between the side injected with Xhairy2-MO and the uninjected side at stage 13. D: The number of actively cycling cells is decreased in the neural crest region injected with Xhairy2-MO (red arrowhead), compared with the uninjected side at stage 18. **E–H:** TUNEL staining of Xhairy2-MO or Control-MO injected embryos. E,G: There is no detectable difference in the number of apoptotic cells between the side injected with Control-MO and the uninjected side at stages 13 (E) and 18 (G). F: There is no detectable difference in the number of apoptotic cells between the side injected with Xhairy2-MO and the uninjected side at stage 13. H: A significant increase in the number of apoptotic cells is observed on the side injected with Xhairy2-MO (red arrowhead), compared with the uninjected side at stage 18. Dorsal views with anterior to the bottom. Injected area is the right side.

crest. In contrast, several recent studies support the idea that BMPs set up a competency zone for the neural crest (reviewed by Raible, 2006). Therefore, it is interesting to speculate that *Xhairy2* may function to confer "competence" for neural crest induction downstream of the BMP signaling pathway, and the gene regulatory network can work only in cells expressing *Xhairy2*.

As only the molecular mechanism(s) for the direct induction of the neural crest have been examined so far, it is important to study the molecular nature of this competence for neural crest induction.

EXPERIMENTAL PROCEDURES

Plasmid Construction

For overexpression studies, we used *Xhairy2b* and *p27^{xic1}*. *pCS2AT-Xhairy2b* was constructed as described previously (Yamaguti et al., 2005). For the construction of *pCS2AT-p27^{xic1}*, oligonucleotides were designed (5′CCCCGAATTCCACCATGGCTGCTTTCCACATCGCCCTG3′ and 5′CCCCGGCGCGCCTCATCGAATCTTTTTCCTGGGGGT3′). The annealed oligonucleotides were subcloned into the EcoRI/Asc1 site of pCS2AT+ that was constructed by inserting annealed oligonucleotides (5′TCGAGGGCGCGCCGATATCTCTAGACGCCCTATAGTGAGTCGTATTAC3′ and 5′GTAATACGACTCACTATAGGGCGTCTAGAGATATCGGCGCGCCC3′) into XhoI-SnaBI-digested pCS2+. This strategy creates new Asc1 EcoRV sites in the polylinker 1 region. The expression plasmid for *X-Delta-1*^{stu} was described previously (Chitnis et al., 1995).

Embryo Manipulation and Injection

Xenopus embryos were in vitro fertilized and cultured as described (Hawley et al., 1995). Embryos were staged according to Nieuwkoop and Faber (1967), and fixed in MEMFA (Harland, 1991). For microinjection studies, synthetic capped mRNAs (produced using mMESSAGE mMACHINE Kit; Ambion) and Morpholino oligonucleotides (MOs; generated by Gene Tools) were injected into the dorsal animal blastomere of eight-cell-stage embryos. For synthesis of mRNAs, each construct was linearized by Not1 and transcribed with SP6 RNA polymerase (Ambion). MOs were resuspended in sterile water to a concentration of 0.1 mM each, and 4 or 8 nl of these solutions was injected. Xhairy2a-MO, Xhairy2b-MO, five-mismatch Xhairy2a-MO, five-mismatch Xhairy2b-MO, and p27^{xic1}-MO have been previously published (Yamaguti et al., 2005; Murato et al., submitted; Vernon et al., 2003). Xhairy2a and Xhairy2b are likely to represent alternative copies of the same gene (Tsuji et al., 2003). The percentage of repressed early neural crest marker expression by Xhairy2b-MO is higher than that by Xhairy2a-MO (Murato et al., submitted). In this study, Xhairy2-MO is a 1:1 mixture of Xhairy2a-MO and Xhairy2b-MO, and Control-MO is a 1:1 mixture of five-mismatch Xhairy2a-MO and five-mismatch Xhairy2b-MO. The standard control morpholino oligonucleotide 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (Gene Tools) was used as negative control of p27^{xic1}-MO. To confirm the injected region, β-galactosidase (β-gal) and yellow fluorescent protein (YFP) mRNA were co-injected. β-gal activity of nuclear lacZ was visualized with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Wako).

Whole-Mount In Situ Hybridization and Probe Preparation

Whole-mount in situ hybridization was performed essentially as described (Harland, 1991). Double in situ hybridization was performed as described (Takada et al., 2005). Antisense RNA probes were synthesized by in vitro transcription in the presence of digoxigenin-UTP (Roche) or fluorescein-UTP (Roche). Alkaline phosphatase substrates used were nitroblue tetrazolium/5-bromo-4-chloro-3-indoxyl phosphate (NBT/BCIP, Roche), BCIP, or 5-bromo-6-chloro-3-indoxyl phosphate (BCIP RED, Biotium).

Antisense RNA probes for in situ hybridization were prepared from templates encoding *Xhairy2b* (Tsuji et al., 2003), *p27^{xic1}* (Su et al., 1995), *n-tubulin* (Chitnis et al., 1995), *Sox2* (Mizuseki et al., 1998), *X-Delta-1* (Chitnis et al., 1995), *Keratin* (Jonas et al., 1985), *X-ngnr-1* (Ma et al., 1996), *FoxD3* (Sasai et al., 2001), *Slug* (Mayor et al., 1995), *Xtwist* (Hopwood et al., 1989), and *Xbmp4* (Hemmati-Brivanlou and Thomsen, 1995).

Proliferation and TUNEL Assay

Phosphohistone H3 immunohistochemistry.

For phosphohistone H3 detection, embryos were fixed in MEMFA, washed with PBS containing 0.1% Triton X-100 (PBT), and blocked in PBT containing 10% heat-treated goat serum. Anti-phosphohistone H3 antibody (Upstate Biotechnology) was used at a concentration of 5 μg/ml. Anti-rabbit IgG conjugated with alkaline phosphatase (Chemicon, 1:1,000) was used as a secondary antibody, and detected with NBT/BCIP.

TUNEL staining was performed as previously described (Hensey and Gautier 1998). In brief, fixed embryos were rehydrated in PBS containing 0.1% Tween 20, and washed with terminal deoxynucleotidyl transferase (TdT) buffer (Gibco) for 30 min. End labeling was carried out at room temperature overnight in TdT buffer containing 2 μM digoxigenin-11-dUTP (Roche) and 150 U/ml TdT (Gibco). Embryos were washed at 65°C with PBS/1 mM EDTA for 1 h, and detected with NBT/BCIP.

Cartilage Staining

For cartilage staining, embryos were fixed in MEMFA at stage 45, washed with PBS, and stained overnight with 0.2% alcian blue/30% acetic acid in ethanol. Embryos were washed with 80% glycerol/2% KOH.

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