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RESEARCH ARTICLE

Δ*Np63* Is Regulated by BMP4 Signaling and Is Required for Early Epidermal Development in *Xenopus*

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Background: It has been established in several models that the *p63* gene has an important role in the development of the epidermis and its derivatives. In *Xenopus*, only the ΔNp63 isoform of this gene has been cloned and its role during epidermal development remains unknown. Results: In this work, we showed that Δ*Np63* is expressed in the nonneural ectoderm since the gastrula stage and that it is regulated by the bone morphogenetic protein 4 (BMP4) signaling pathway. Our in vivo and in vitro experiments demonstrated that Δ*Np63* is required in the earliest inductive steps of epidermal development. The overexpression of Δ*Np63* caused an increase in epidermal markers with a suppression of neural induction while the blocking of Δ*Np63* led to the opposite results. Finally, we found that Δ*Np63* acts as an anti-apoptotic gene, regulating the transcription of some apoptotic and anti-apoptotic factors. Conclusion: The results suggest that Δ*Np63* is an essential gene in early epidermal specification under the control of BMP4. *Developmental Dynamics 241:257–269, 2012.* © 2011 Wiley Periodicals, Inc.

Key words: epidermis; specification; BMP4; Δ*Np63*; apoptosis

Key findings:

- Δ*Np63* expression is regulated by BMP signaling.
- Gain- and loss-of-function experiments show that Δ*Np63* is essential since the earliest steps of epidermis induction for the proper development of this tissue.
- Δ*Np63* participates in epidermal development regulating the expression of some components of the apoptotic pathway.

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INTRODUCTION

In vertebrates, ectodermal cells first choose between two fates, neural or epidermal. Epidermal development implicates different stages such as epidermal induction, specification, differentiation, and stratification that are regulated by multiple mechanisms (Sasai and De Robertis, 1997; Koster and Roop, 2004; Chalmers et al., 2006). Epidermis development bears many conserved features between mammals and amphibians in late organogenesis, but they appear to be significantly different in the initial stages of epidermis development (Ma, 2009). The molecular mechanisms of early epidermal development are probably better known in the frog embryo. It is accepted that the initial specification of *Xenopus* epidermis is

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controlled by a bone morphogenetic protein (BMP) activity gradient (Wilson and Hemmati-Brivanlou, 1995; Wilson et al., 1997; LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Tribulo et al., 2003). A high BMP activity is able to promote epidermal fate (Wilson and Hemmati-Brivanlou, 1995) by activating some transcription factors such as *msx1*, *vent2*, *Dlx*, *AP2α*, *Xfoxi1a*, and *Grainyhead like-1* (Onichtchouk et al., 1996; Suzuki et al., 1997b; Luo et al., 2001, 2002; Tao et al., 2005), which act both promoting epidermal development and inhibiting neural fate.

The transcription factor *p63*, a member of the *p53* gene family, plays a fundamental role in the development of mammalian and zebrafish epidermis. In mammals, six isoforms of *p63* are produced by the activity of two alternative promoters (TA and ΔN) and alternative splicing (α, β, and γ). The transactivating (TA) isoforms contain an amino-terminal exon that encodes a transactivation domain, whereas Δ*Np63* isoforms lack this domain but conserve the common DNA binding (DBD) and oligomerization (OD) domains. Alternative splicing at the 3′ end generates TA and ΔNp63 proteins with different C-termini, termed α, β and γ (Yang et al., 1998). Several studies have proposed that Δ*Np63* has a dominant-negative function and acts as a transcriptional repressor (Yang et al., 1998; Westfall et al., 2003; Tomimori et al., 2004; Barton et al., 2009). However, Δ*Np63* was also shown to be able to transactivate target gene expression (Wu et al., 2003; King et al., 2006; Koster et al., 2007; Radoja et al., 2007; Romano et al., 2009). Although the *p63* sequence is well conserved in vertebrates, only ΔN isotypes have been identified in zebrafish (Bakkers et al., 2002; Lee and Kimelman, 2002), chick (Yasue et al., 2001), and *Xenopus laevis* (Lu et al., 2001; Tomimori et al., 2004).

Experimental evidence from transgenic mice and mouse embryonic stem (ES) cells models strongly suggest that Δ*Np63* is required for both epidermal commitment to stratification (Koster et al., 2004; Medawar et al., 2008) and epidermal stem cell/progenitor cell self renewal (Senoo et al., 2007). In mice embryos, it has been reported that Δ*Np63* is strongly expressed in the basal layer of the epidermis where highly proliferative epithelial stem cells reside, and that this gene is able to promote the viability and proliferation of this cell type (Westfall et al., 2003; Koster et al., 2004; Barbieri et al., 2005; Candi et al., 2006; Truong et al., 2006). In addition, Δ*Np63* down-regulation causes severe skin erosions by impairing terminal differentiation and basement membrane formation (Koster et al., 2007). In zebrafish embryos, it was shown that Δ*Np63* is a direct target of BMP signaling and is essential for ventral ectoderm specification in early embryos as well as in epidermal cell proliferation at later developmental stages (Bakkers et al., 2002; Lee and Kimelman, 2002). Data gathered so far suggest that Δ*Np63* plays critical roles in the development, maintenance, and differentiation of the epidermis but also reveal that the timing of expression and functions of this gene in mammals could be different from that of fishes. Although it was suggested that Δ*Np63* suppresses mesodermal cell fate during *Xenopus* early development (Barton et al., 2009), the role of Δ*Np63* in the induction and differentiation of epidermal cells remains unknown.

Apoptosis is required for normal embryogenesis (Baehrecke, 2002) and may be an important mechanism involved in epithelial development (Borrelli et al., 2009). It has been found that, apart from enhancing cell proliferation, Δ*Np63* inhibits apoptosis (Patturajan et al., 2002; Lee et al., 2006). In agreement with the above, recent studies have shown that Δ*Np63* regulates the pro-apoptotic genes *Bax* and *Apaf1* in cancer and ventral bladder development (Cheng et al., 2006; Mundt et al., 2010).

In the present study, our goal was to gain an insight into the role of Δ*Np63* during the early induction and specification of *Xenopus* epidermis. We report here that Δ*Np63* is expressed in the nonneural ectoderm since the early stages of epidermis development, in the innermost layer of the prospective epidermis, and that its expression is regulated in a BMP4-dependent manner. We found that Δ*Np63* mediates proper ectodermal development by promoting epidermal induction and inhibiting neural tissue development. Finally, we demonstrate that Δ*Np63* overexpression inhibits apoptosis both in vivo and in vitro.

Fig. 1. Analysis of Δ*Np63* expression in developing embryos. **A:** Analysis of Δ*Np63* temporal expression pattern. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on total RNA extracted from embryos at different embryonic stages. *EF1α* amplification was used as control. B–P: Whole-mount in situ hybridization analysis of spatiotemporal Δ*Np63* expression. **B:** Lateral view. **C,D,J–L:** Lateral view, anterior on the right, dorsal at the top. **E,M–P:** Anterior view, dorsal at the top. **F,H,I:** Dorsal view, anterior on the right. **G,M′–P′**: Transversal sections. B,F,G,H: In situ hybridization for Δ*Np63*. C,D: Double in situ hybridization for Δ*Np63* (purple) and *XK81a* (turquoise). G: Small arrow, Δ*Np63* expression in the internal layer of the ectoderm. Inset, Δ*Np63* (purple) and *XK81a* (turquoise). (H) Small arrow, two medial stripes of Δ*Np63* expression. E,N,N′: Δ*Np63* (purple) and *Sox2* (turquoise). I,J,M,M′: Δ*Np63* (purple) and *FoxD3* (turquoise). L,P,P′: Δ*Np63* (purple) and *Dlx5* (turquoise). K, O, O′: In situ hybridization for *Six1*. Asterisk, Δ*Np63* expression in the prospective epidermis. Red star, ventral anterior prospective epidermis with no Δ*Np63* expression. Arrowhead, Δ*Np63* expression in the most external limit of the neural crest overlapping with *Six1* and *Dlx5* expression. Large arrow, anterior Δ*Np63* expression overlaps with *Six1* and *Dlx5*. i.l., internal layer; e.l., external layer. An, animal; Vg, vegetal; D, dorsal; V, ventral; np, neural plate.

Fig. 2. Δ*Np63* expression is decreased by inhibiting bone morphogenetic protein (BMP) signaling. A,A′,B,B′: One dorsal blastomere of 8- to 16-cell stage embryos was injected with 500 pg of *CM-BMP4* (A,A′) or *dnBMPR* mRNA (B,B′) and the expression of Δ*Np63* was analyzed at stage 16. **A,B:** Dorsal view, anterior on the right. **A′,B′:** Anterior view, dorsal at the top. The injected side was recognized by FLDx staining (A,A′) or fluorescence (B,B′) and is indicated by an arrowhead. **C–F:** One-cell stage embryos were injected with *CM-BMP4* (D,F) or *dnBMPR* mRNA (E). Control embryos were not injected. At stage 9, animal caps were dissected and processed for reverse transcriptase-polymerase chain reaction (RT-PCR) or in situ hybridization when sibling embryos reached neurula stage 16. C–E: Note the reduced Δ*Np63* expression analyzed by in situ hybridization in injected animal caps (D,E) compared with control caps (C). F: Total RNA was isolated from stage 14 embryos and treated and control caps and the expression of Δ*Np63*, epidermal markers *XK81a* and *Dlx3*, neural marker *Sox2* and the mesodermal marker *XBra* was analyzed by RT-PCR. *EF1α* amplification was used as control.

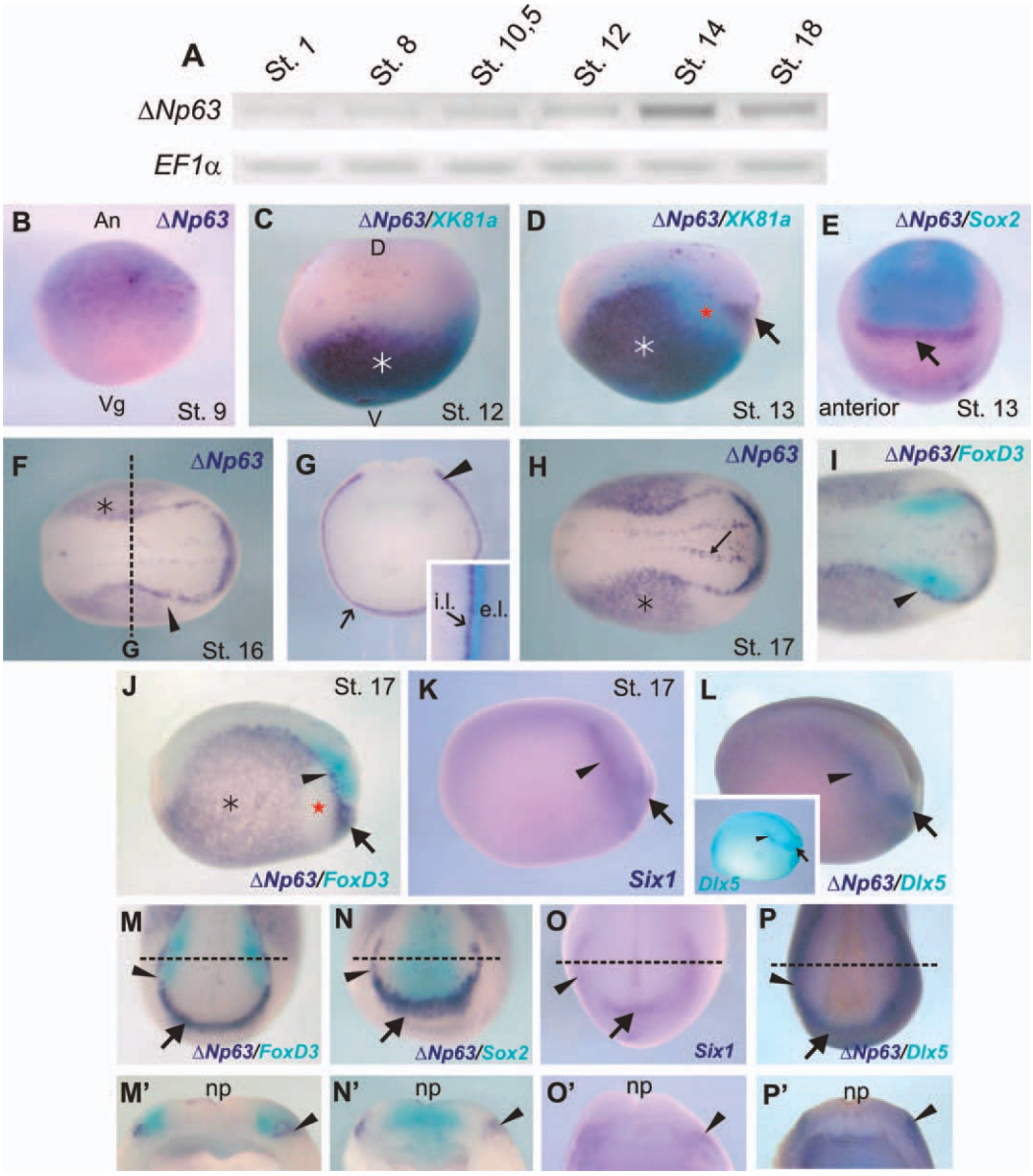
A
St. 1
St. 8
St. 10,5
St. 12
St. 14
St. 18
ΔNp63
EF1α
B An ΔNp63 Vg St. 9
C ΔNp63/XK81a D V St. 12
D ΔNp63/XK81a St. 13
E ΔNp63/Sox2 anterior St. 13
F ΔNp63 G St. 16
G i.l. e.l.
H ΔNp63 St. 17
I ΔNp63/FoxD3
J St. 17 ΔNp63/FoxD3
K St. 17 Six1
L Dlx5 ΔNp63/Dlx5
M ΔNp63/FoxD3
N ΔNp63/Sox2
O Six1
P ΔNp63/Dlx5
M' np
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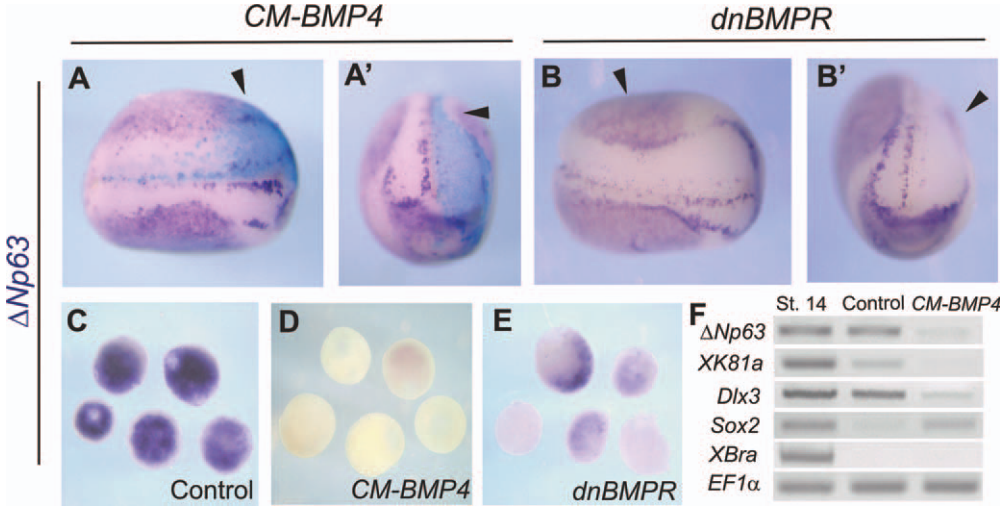
Fig. 1.


CM-BMP4
dnBMPR
ΔNp63
A
A'
B
B'
C Control
D CM-BMP4
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ΔNp63
XK81a
Dlx3
Sox2
XBra
EF1α

Fig. 2.

RESULTS

Xenopus Δ*Np63* Is Expressed in the Nonneural Ectoderm

To explore the role of Δ*Np63* during the early development of the epidermis, we first analyzed its expression pattern by reverse transcriptase-polymerase chain reaction (RT-PCR; Fig. 1A) and whole-mount in situ hybridization (Fig. 1B–O). Weak maternal expression of Δ*Np63* was detected by RT-PCR. Δ*Np63* was up-regulated during gastrulation, and its expression was maintained in the subsequent stages (Fig. 1A). The analysis of temporospatial Δ*Np63* expression was performed by single and double in situ hybridization since the early developmental stages to find out the onset of its expression, which was still unknown for *Xenopus* embryos. We also performed a detailed comparison between Δ*Np63* expression and different neural, epidermal and placodal marker genes. This enabled us to delimit the precise boundaries of Δ*Np63* expression in the ectoderm. Δ*Np63* was widely expressed in the animal cap region at the blastula stage (stage 9, Fig. 1B). From the late gastrula stage (stage 12) onward, the expression of Δ*Np63* was restricted to the nonneural ectoderm, displaying an expression pattern partially overlapping with the epidermal marker *XK81a* (Fig. 1C,D, asterisk). A small region in the ventral anterior prospective epidermis expressing *XK81a* but not Δ*Np63* was observed (Fig. 1D, red star). At the early neurula stage, the epidermis expressed higher levels of Δ*Np63* (Fig. 1D) and its expression was also observed as a strong line in the most anterior limit between epidermis and neural plate (Fig. 1D,E, arrow). Double in situ hybridization showed that this anterior expression does not overlap with the neural plate territory expressing the *Sox2* marker (Fig. 1E), indicating that Δ*Np63* expression is located in the cephalic nonneural ectoderm. As development progresses, the Δ*Np63* expression remains located in the prospective epidermis (Fig. 1F,H, asterisk). Transversal sections revealed that Δ*Np63* expression is restricted to the internal layer of the prospective epidermis (Fig. 1G) while *XK81a* expression is located in the external layer (Fig. 1G, inset). The anterior line of expression of Δ*Np63* extends laterally and posteriorly, limiting at the anterior region of the embryo with the neural plate (*Sox2*, Fig. 1N, large arrow) and at the lateral external border with the neural crest (*FoxD3*, Fig. 1I,J,M, arrowhead). This crescent-shaped expression of Δ*Np63* overlapped with *Dlx5* (Fig. 1L,P, large arrow and arrowhead). This expression, which corresponds to the preplacodal territory, was evident also by the comparison between Δ*Np63* and *Six1* expressions (Fig. 1J,K,M,O, arrowhead and large arrow). During the mid-neurula stage, the ventrolateral anterior epidermis region that expresses *XK81a* but not Δ*Np63* persists (Fig. 1J, red star). Although the neural plate does not express Δ*Np63*, two medial rows of expression that extend anteriorly were detected (Fig. 1H, small arrow). Transversal sections showed the defined expression in the external limit of the neural crest that is complementary with the expression of the neural crest marker *FoxD3* (Fig. 1M′) and overlaps with the placodal markers *Six1* (Fig. 1O′) and *Dlx5* (Fig. 1P′). These results demonstrate that Δ*Np63* expression is mainly located outside the neural plate and the neural crest domains, in the innermost layer of the prospective epidermis and in the preplacodal ectodermal domain.

Control of Δ*Np63* Expression by BMP Signaling

Previous reports in zebrafish and mouse ES cells showed that Δ*Np63* is a direct target of BMP signaling (Bakkers et al., 2002; Barton et al., 2009). We wondered whether an active BMP4 signaling pathway was necessary for Δ*Np63* expression in *Xenopus* embryos. To assess this, we expressed a dominant-negative truncated BMP4 (*CM-BMP4*) or a dominant-negative BMP4-specific receptor (*dnBMPR*; Hawley et al., 1995) in developing embryos. The injection of one dorsal blastomere of 8- to 16-cell stage embryos produced an inhibition in the Δ*Np63* expression domain with both *CM-BMP4* (Fig. 2A,A′; 63%, n = 45) and *dnBMPR* (2B,B′; 56%, n = 74). The effect was evidenced by a decrease in Δ*Np63* expression not only in the epidermis but also in the external limit of the neural crest (Fig. 2A,B) and in the most anterior region between neural plate and epidermis (Fig. 2A′,B′). An expansion in the neural plate domain was also observed (Fig. 2A,B). We then performed experiments using ectodermal explants (animal cap assay) to distinguish direct effects on the ectoderm from secondary effects by means of the mesoderm. *CM-BMP4* or *dnBMPR* mRNAs were injected into one-cell stage embryos. Control embryos were not injected. When control and injected embryos reached stage 9, ectodermal explants were dissected out and cultured until the equivalent of stage 16. Then, the expression of Δ*Np63* in animal caps was analyzed by in situ hybridization of treated and control explants. Δ*Np63* expression was diminished by *CM-BMP4* (Fig. 2D; 85%; n = 50) and *dnBMPR* injections (Fig. 2E; 74%; n = 46) compared with controls (Fig. 2C). RT-PCR analysis of explanted animal caps injected with *CM-BMP4* revealed an up-regulation of *Sox2* consistent with the neuralization effect and a reduction in Δ*Np63* concomitant with the decrease in epidermal markers *Dlx3* and *XK81a* (Fig. 2F). The mesodermal marker *XBra* was not detected, which indicates that the neuralizing effect of CM-BMP4 is independent of the mesoderm. Taken together, our results strongly support the idea that Δ*Np63* expression in *Xenopus* embryos is dependent on the activity of BMP signaling.

Δ*Np63* Promotes Epidermal Differentiation

The restricted Δ*Np63* expression pattern in addition to its regulation by BMP4 signaling suggests that this gene is involved in epidermal development. To further analyze this question, we performed Δ*Np63* gain-of-function experiments. The injection of Δ*Np63* mRNA into a dorsal blastomere of 16-cell stage embryos led to an expansion of the epidermal territory evidenced by an expanded and increased expression of the epidermal markers *XK81a* (Fig. 3A; 65%, n = 60), *Dlx3* (Fig. 3B; 83%; n = 78), and *Rexp52* (Fig. 3C; 56%;

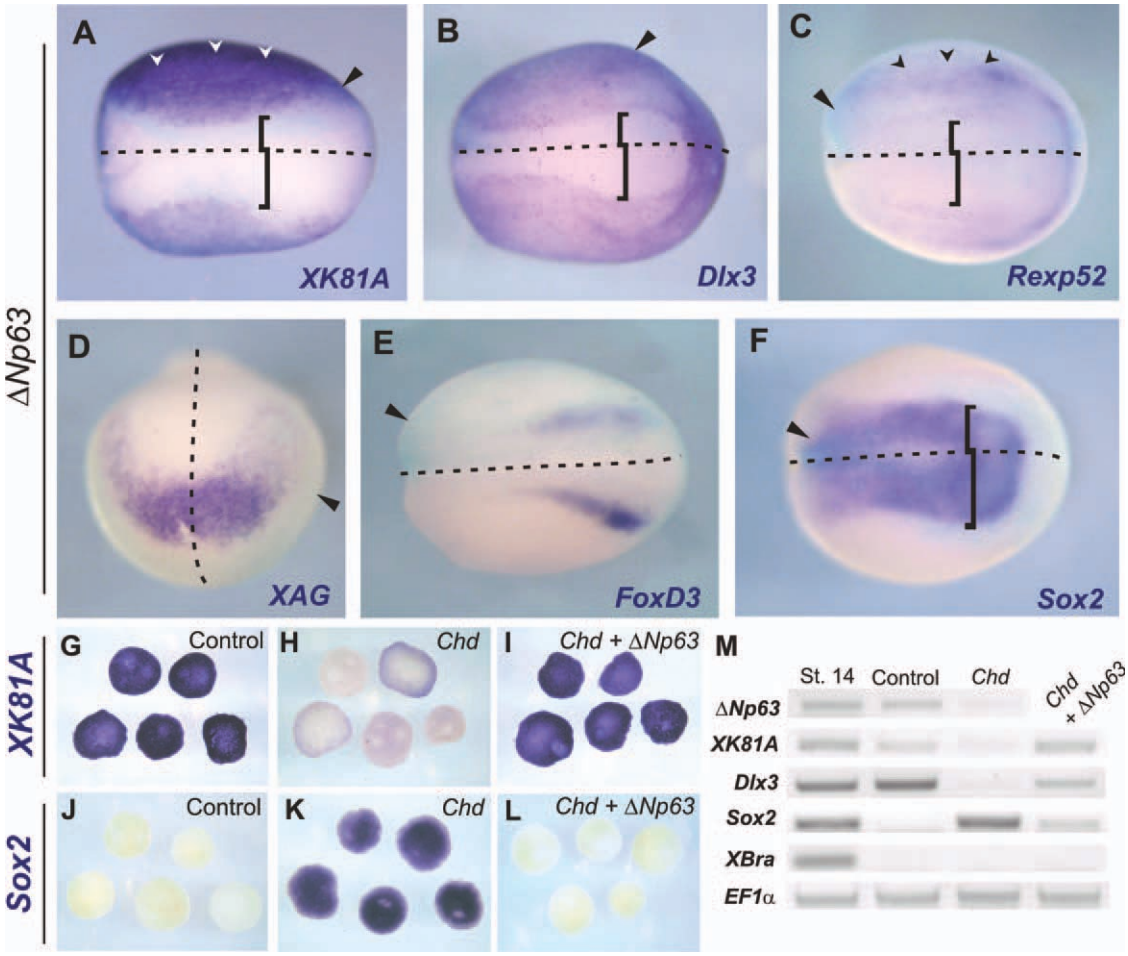


Fig. 3. Overexpression of Δ*Np63* induces epidermal fate and suppresses neural induction. A–F: Δ*Np63* mRNA (700 pg) was injected into one dorsal blastomere of 16-cell stage embryos. Embryos were fixed at stage 16 and whole-mount in situ hybridization was performed for (A) *XK81a*, (B) *Dlx3*, (C) *Rexp52*, (D) *XAG*, (E) *FoxD3*, and (F) *Sox2*. **A–C,E,F:** Dorsal view, anterior on the right. **D:** Anterior view, dorsal at the top. Dashed lines indicate the midline, small arrowheads indicate increased expression, and brackets indicate the width of the neural plate. Arrowheads indicate the injected side, recognized by FLDx staining. **G–L:** One-cell stage embryos were injected with 500 pg of *Chd* (H,K) or 500 pg of *Chd* + 500 pg Δ*Np63* mRNA (I,L). G,J: Control embryos were not injected. Embryos were cultured until the blastula stage when animal caps were dissected out and processed when sibling embryos reached the equivalent of stage 16. The expression of *XK81a* and *Sox2* was assessed by in situ hybridization. **M:** Total RNA was isolated from treated and control caps and the expression of Δ*Np63*, epidermal markers *XK81a* and *Dlx3*, neural marker *Sox2* and the mesodermal marker *XBra* was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR).

n = 63), a marker of epidermis inner layer (Pollet et al., 2005). In addition, we observed an increase in the expression of the cement gland marker *XAG* (Fig. 3D; 54%; n = 45). In contrast, a significant reduction in *FoxD3* (Fig. 3E; 80%; n = 70) and *Sox2* (Fig. 3F; 75%, n = 82) was observed in the injected side. The results from these in vivo experiments demonstrated that Δ*Np63* was able to up-regulate non-neural ectodermal genes.

To assess whether Δ*Np63* was able to induce epidermis in neuralized animal caps we carried out rescue experiments as follows. One-cell stage embryos were injected with *chordin* (*Chd*) mRNA or *Chd* mRNA plus Δ*Np63* mRNA. Noninjected embryos were used as controls. Embryos were cultured until the blastula stage and then animal caps were dissected out. Explants were cultured and the expression of *XK81a* and *Sox2* was assessed by in situ hybridization when siblings reached the equivalent of stage 16 (Fig. 3G–L). The co-injection of Δ*Np63* rescued the expression of *XK81a* (Fig. 3I; 98%; n = 65) that was inhibited by *Chd* (Fig. 3H; 87%; n = 74). In addition, the *Chd*-induced *Sox2* expression (Fig. 3K; 56%; n = 78) was suppressed by Δ*Np63* (Fig. 3L; 76%; n = 62). Similar results were achieved when the expression of several gene markers was analyzed by RT-PCR (Fig. 3M). When BMP signaling was suppressed by *Chd*, there was ectoderm neuralization evidenced by *Sox2* induction. Under these conditions, a down-regulation of the epidermal marker genes including Δ*Np63* (lane 3) was observed, reinforcing the notion that Δ*Np63* is regulated by BMP signaling. Neural differentiation caused by *Chd* was suppressed by Δ*Np63* overexpression that activated the expression of *XK81a* and *Dlx3* and suppressed the expression of *Sox2* (lane 4). As expected, the expression of the mesodermal marker *XBra* was not modified. Taken together, our in vivo and in vitro results indicate that Δ*Np63* promotes epidermal differentiation at the expense of neural induction.

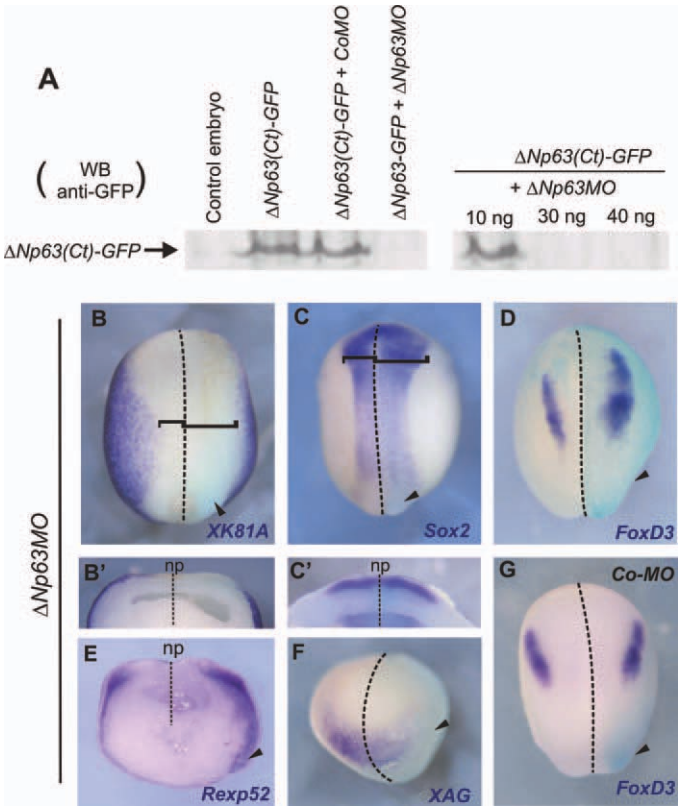
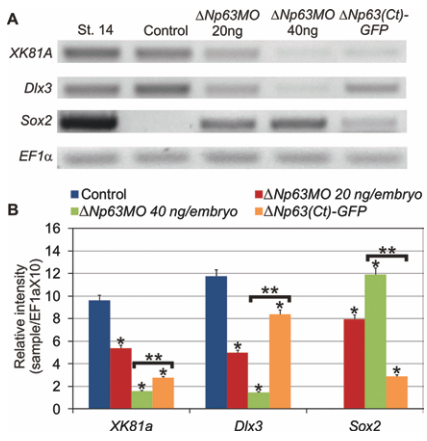


Fig. 4.



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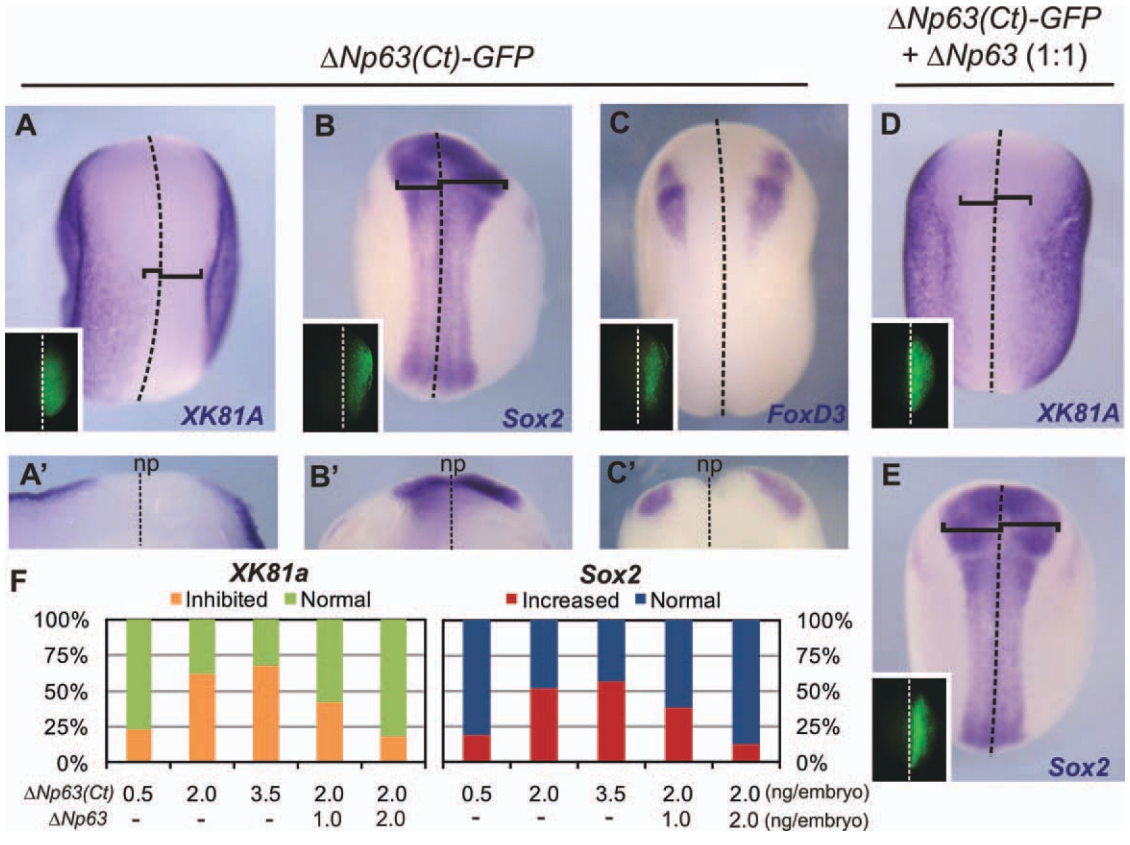

Fig. 5.

ΔNp63 Is Required for Epidermal Gene Expression

The findings presented above support a positive role of Δ*Np63* in the regulation of epidermal gene expression. To further understand the role of this gene, loss-of-function experiments were carried out. Two different approaches were used to interfere with Δ*Np63* function by means of different mechanisms. One of them consisted in the microinjection of an antisense morpholino oligonucleotide (Δ*Np63MO*) and the other in the overexpression of a C-terminally truncated form of Δ*Np63* fused to green fluorescent protein (GFP; named Δ*Np63(Ct)-GFP*). Δ*Np63(Ct)-GFP* lacks the Δ*Np63* oligomerization domain and may act as a dominant negative. To test the efficiency and specificity of Δ*Np63MO*, we used a GFP-tagged form of Δ*Np63*, and we first demonstrated that Δ*Np63MO* was able to inhibit the translation of Δ*Np63(Ct)-GFP* in a dose-dependent manner (Fig. 4A). Embryos at the 16-cell stage were injected into one dorsal blastomere with Δ*Np63MO* (40 ng/embryo; Fig. 4B–F) or an equivalent amount of a control morpholino (*CoMO*; Fig. 4G). The injection of Δ*Np63MO* inhibited the expression of *XK81a* (Fig. 4B,B′; 86%; n = 82), *Dlx3* (79%; n = 43; data not shown) and, of interest, caused a noticeable disruption in the *Rexp52* expression (Fig. 4E; 80%; n = 73). The expression of the cement gland marker *XAG* was also inhibited (Fig. 4F; 62%; n = 35). Conversely, the expression of the neural plate and neural crest markers *Sox2* (Fig. 4C,C′; 57%; n = 46) and *FoxD3* (Fig. 4D; 66%; n = 67) increased in the injected side. Embryos injected with *CoMO* showed normal morphology and expression of *FoxD3* (Fig. 4G).

To assess the role of Δ*Np63(Ct)-GFP* as a dominant negative, different amounts of this construct were injected into one dorsal blastomere of 8-cell stage embryos and the expression of different markers was analyzed at stage 16. The injection of Δ*Np63(Ct)-GFP* mRNA (3.5 ng/embryo) decreased *XK81a* (Fig. 5A,A′, F; 68%; n = 55) and augmented the expression of *Sox2* (Fig. 5B,B′, F; 55%; n = 40) and *FoxD3* (Fig. 5C,C′; 58%; n = 46). The percentage of embryos that presented altered phenotypes was higher as we increased the dose (Fig. 5F). These phenotypes were similar to those produced by Δ*Np63MO*. These findings could indicate that Δ*Np63(Ct)-GFP* is acting in a dominant negative way. To show that Δ*Np63(Ct)-GFP* specifically inhibits Δ*Np63* function, we performed rescue experiments. The injection of 2 ng/embryo of Δ*Np63(Ct)-GFP* inhibited the expression of *XK81a* in 62% of the embryos (Fig. 5F; n = 45) and increased the expression of *Sox2* in 52% of the embryos (Fig. 5F; n = 52). However, the co-injection of Δ*Np63(Ct)-GFP* and Δ*Np63* was able to rescue the expression of *XK81a* and *Sox2* in a dose-dependent manner. When Δ*Np63(Ct)-GFP* was co-injected with Δ*Np63* at a 2:1 ratio, the inhibition in the expression of *XK81a* was reduced to 42% (Fig. 5F; n = 77) and the increase in *Sox2* was reduced to 38% of the embryos (Fig. 5F; n = 60), while equal amounts of Δ*Np63(Ct)-GFP* and Δ*Np63* (1:1) rescued the expression of *XK81a* (Fig. 5D,F; n = 65) and *Sox2* (Fig 5E,F; n = 73) and less than 20% of embryos presented altered phenotypes. These results allow us to conclude that the phenotypes produced by Δ*Np63(Ct)-GFP* lead to a modulation of effects by competition with Δ*Np63* target genes.

We next carried out in vitro experiments to further study the requirement of Δ*Np63* during epidermal induction. We injected two different amounts of Δ*Np63MO* (20 and 40 ng/embryo) or Δ*Np63(Ct)-GFP* mRNA (3.5 ng/embryo) into one-cell stage embryos and dissected out ectodermal explants as described above. In agreement with the in vivo results, RT-PCR experiments performed on ectodermal explants showed that Δ*Np63MO* and Δ*Np63(Ct)-GFP* significantly down-regulate *XK81a* and *Dlx3* and increase *Sox2* expression (Fig. 6A, lanes 3–5).

Fig. 4. Δ*Np63* is required for epidermal initial development. **A:** Both blastomeres of two-cell stage embryos were injected with Δ*Np63(Ct)-GFP* mRNA (3.5 ng/embryo). Δ*Np63(Ct)-GFP* was coinjected with *CoMO* (40 ng/embryo) or with Δ*Np63MO* (10, 30 or 40 ng/embryo). Injected and control embryos were cultured until stage 16 and processed for total protein extraction. Δ*Np63(Ct)-GFP* protein was detected by western blotting using an anti-green fluorescent protein (GFP) antibody. Each lane was loaded with 100 μg of total protein. Δ*Np63MO* inhibits translation of Δ*Np63* in a dose dependent manner. **B–G:** Δ*Np63MO* (40 ng/embryo; B–F) or *CoMO* (40 ng/embryo; G) was injected into one blastomere of 8- to 16-cells stage embryos. Embryos were fixed at stage 16, and the expression of different genes was analyzed by in situ hybridization. B–D,G: Dorsal view, anterior at the top. F: Anterior view, dorsal at the top. B′,C′,E: Transverse sections. Arrowhead indicates the injected side evidenced by the linage tracer FLDx. Dashed lines indicate the midline. Brackets indicate the width of the neural plate. B,E,F: Δ*Np63*-depleted embryos fail to correctly express epidermal markers *XK81a* (B), *Rexp52* (E), and cement gland marker *XAG* (F). C,D: Neural plate marker *Sox2* and neural crest marker *FoxD3* are expanded in the Δ*Np63MO*-injected side of embryos. F: Injection of control morpholino (*CoMO*) showed no effect on *FoxD3* expression. np, neural plate.

Fig. 5. Δ*Np63(Ct)-GFP* acts as a dominant negative of Δ*Np63*. A–F: One dorsal blastomere of eight-cell stage embryos was injected with 0.5 ng (F), 2.0 ng (F), or 3.5 ng (A–C,F) of Δ*Np63(Ct)-GFP* mRNA or with different combinations of Δ*Np63(Ct)-GFP* and Δ*Np63* (D–F). Embryos were fixed at stage 16 and the expression of several genes was analyzed. The injected side is evidenced by green fluorescent protein (GFP) fluorescence. **A–C:** Dorsal view, anterior at the top. A′–C′: Transverse sections. Dashed lines indicate the midline of embryos. Brackets indicate the width of the neural plate. The injected side of the embryos treated with Δ*Np63(Ct)-GFP* shows a reduction in *XK81a* expression (A,A′) and an increase in *Sox2* (B,B′) and *FoxD3* (C,C′) expression. **D,E:** The coinjection of Δ*Np63(Ct)-GFP* and Δ*Np63* (1:1 ratio) rescued *XK81a* (D) and *Sox2* (E) expressions. **F:** Quantification of Δ*Np63(Ct)-GFP* dose effects and rescue experiments. np, neural plate.

Fig. 6. Inhibition of Δ*Np63* leads to a decrease in epidermal markers expression. **A:** Animal caps were dissected from embryos injected at one blastomere stage with Δ*Np63MO* (20 and 40 ng/embryo) or Δ*Np63(Ct)-GFP* (3.5 ng/embryo). Explants were excised from stage 9 embryos and cultured until sibling embryos reached stage 16. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect the epidermal markers *XK81a* and *Dlx3* and the neural marker *Sox2*. *EF1α* was used as amplification control and as housekeeping gene for expression normalization. Total RNA from stage 14 embryos was used as positive control. **B:** Quantification of the gel shown in (A). Results are expressed as Relative Intensity (Sample/EF1αX10). Student's *t*-test was used to analyze the differences between each groups and the corresponding control group. Differences were considered statistically significant at $P < 0.001$(*). Also the effects produced by Δ*Np63(Ct)-GFP* and Δ*Np63* (40 ng/embryo) were statistically different ($P < 0.001$, **).

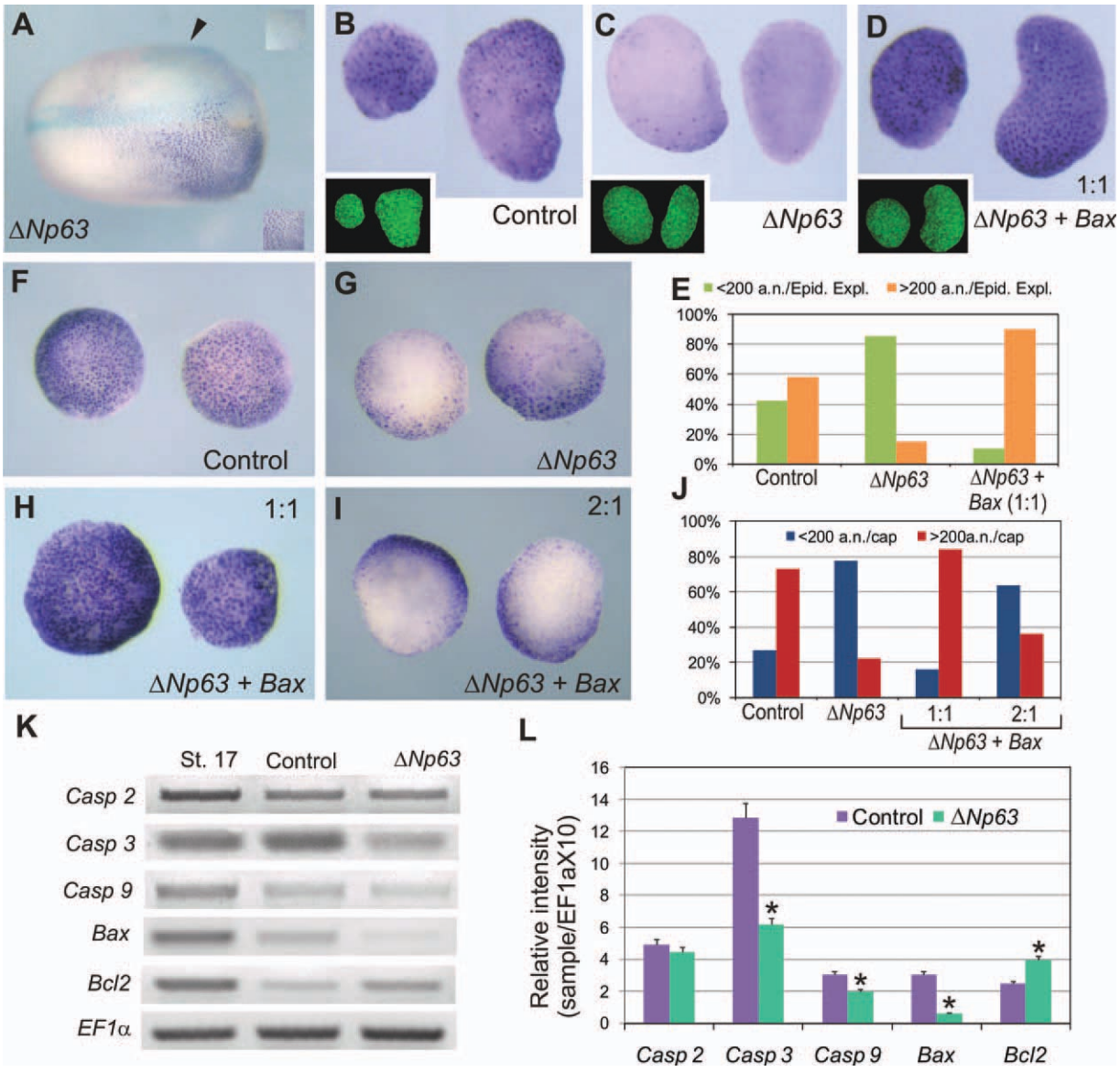

Fig. 7. Δ*Np63* inhibits apoptosis in embryos and animal caps. A–I: One dorsal blastomere of 8-cell stage embryos (A) or one-cell stage embryos (B–I) were injected with 1 ng of Δ*Np63* (A,C,G), 1 ng of Δ*Np63* + 1 ng of *Bax* (C,H), or 1 ng of Δ*Np63* + 500 pg of *Bax* (I). **A:** Embryos were fixed at stage 16 or cultured. **B–D:** A set of embryos was grown until stage 13 and epidermis explants were dissected and cultured until the equivalent of stage 16. **F–I:** Another set was cultured until the blastula stage when animal caps were dissected and cultured until the equivalent of stage 16. Apoptosis was analyzed by terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) staining. A: Dorsal view, anterior on the right. The arrowhead indicates the injected side containing FLDx as lineage tracer. B,C: Inset: FLDx evidenced by fluorescence. **E:** Quantification of the epidermis explants experiments. **J:** Quantification of the animal cap experiments. a.n., apoptotic nuclei. **K:** One-cell stage embryos were injected with 1ng of Δ*Np63* mRNA; control embryos were not injected. Embryos were cultured until the blastula stage when animal caps were dissected out and processed for reverse transcriptase-polymerase chain reaction (RT-PCR). *Caspases 2*, *3*, *9*, *Bax* and *Bcl2* expression was analyzed. *EF1*α was used as a loading control. **L:** Quantification of the gel that is shown in (K). Results are expressed as Relative Intensity (Sample/EF1αX10). Student's *t*-test was used to analyze the differences between each group and the corresponding control group. Differences were considered statistically significant at $P < 0.001$(*).

Moreover, data revealed that the effects produced by Δ*Np63MO* were dose-dependent. Changes in gene expression produced by Δ*Np63(Ct)-GFP* were less marked than those produced by Δ*Np63MO* (Fig. 6B). Collectively, these studies strongly support our model of a key role for Δ*Np63* in the control of early epidermal induction.

Δ*Np63* Overexpression Inhibits Apoptosis In Vivo and In Vitro

There is evidence that suggests that Δ*Np63* plays an anti-apoptotic role during bladder development (Cheng et al., 2006). We, therefore, decided to analyze whether Δ*Np63* participates in epidermal development by controlling apoptosis. To determine apoptosis levels, we performed terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) assays on embryos, ectodermal animal cap explants and in epidermal explants in which Δ*Np63* was overexpressed.

A decrease in the number of TUNEL stained cells was observed in the Δ*Np63*-injected side of embryos (Fig. 7A; 58%; n = 45), epidermal explants (Fig. 7C,E; 85%; n = 66), and animal caps (Fig. 7G, J; 78%; n = 85) compared with controls (Fig. 7B,E; n = 60; Fig. 7F,J; n = 50). The inhibition of apoptosis produced by Δ*Np63* overexpression was reversed by co-injecting the mRNA coding for the pro-apoptotic factor *Bax* in epidermal explants (Fig. 7D, E; 90%; n = 75) and animal caps (Fig. 7H,J; 84%; n = 63). When the ratio of Δ*Np63:Bax* was 2:1, apoptosis levels decreased again in animal caps (Fig. 7I,J; 64%; n = 75), suggesting that Δ*Np63* inhibits apoptosis by negatively regulating *Bax* expression.

To further investigate the role of Δ*Np63* in apoptosis, we used RT-PCR to assess the effect of Δ*Np63* overexpression in animal caps on the expression of several caspases and two *Bcl2* family members (Fig. 7K,L). Δ*Np63* reduced the expression of *caspase 3* and *9* while no effect on *caspase 2* expression was observed (Fig. 7K,L, lane 3). With regard to *Bcl2* family members, Δ*Np63* decreased the levels of *Bax* and increased the expression of the anti-apoptotic factor *Bcl2* (Fig. 7K,L, lane 3). These results support the notion that Δ*Np63* is able to regulate the expression of some components of the apoptotic pathway and, furthermore, suggest that Δ*Np63* participates in epidermal development by inhibiting programmed cell death.

DISCUSSION

The present study support a role for Δ*Np63* as an early player in the genetic cascade that mediates the initial specification of the nonneural ectoderm to become epidermis through BMP signaling. Furthermore, it shows that Δ*Np63* prevents epidermis apoptosis by regulating the mitochondrial apoptotic pathway.

Δ*Np63* Expression Pattern

Δ*Np63* is expressed in epidermal tissue or its derivatives in a wide range of vertebrates. In mouse embryos, Δ*Np63* is expressed since the E9.5 embryonic stage during epidermal (Mills et al., 1999), tooth, and hair development (Laurikkala et al., 2006; Mikkola, 2007; Romano et al., 2007), and its expression co-localizes with the basal markers K5 and K14 during skin development (Romano et al., 2009). In zebrafish, Δ*Np63* expression is first confined to the developing epidermis and later to the ectodermal components of branchial arches and pectoral fin buds (Bakkers et al., 2002; Lee and Kimelman, 2002). Δ*Np63* expression in the chick embryo is detected in the prospective cutaneous ectoderm, apical ectodermal ridge of the limb bud, ectodermal epithelium of branchial arches, and feather buds (Yasue et al., 2001). It should be noticed that these reports showed a Δ*Np63* expression in epidermal tissue or its derivatives comparable to studies performed in *Xenopus* showing that metamorphosis events were preceded by enhanced Δ*Np63* expression in equivalent embryonic tissues (Tomimori et al., 2004).

A previous report by Lu et al. (2001) showed that *Xenopus* Δ*Np63* is expressed in the epidermis from midneurula onward. Here we report for the first time the earliest expression of Δ*Np63* during *Xenopus* embryogenesis. We detected Δ*Np63* expression even before the mid blastula transition, suggesting a maternal origin of the Δ*Np63* mRNA. Moreover, by comparing the expression of several ectodermal marker genes, we revealed a restricted expression of Δ*Np63* in the innermost layer of the presumptive epidermis in addition to a defined expression of this gene in the external limit of the neural crest and in the anteriormost limit between epidermis and neural plate. Our results confirmed that this Δ*Np63* crescent-shaped expression corresponds to the prospective placodal ectoderm (Pandur and Moody, 2000; Luo et al., 2001; Schlosser and Ahrens, 2004). Taken together, our data suggest an earlier role of Δ*Np63* in epidermal and placodal specification.

Δ*Np63* Is a Target of BMP4 Signaling

BMP4 signaling pathway is necessary for differentiation of the embryonic *Xenopus* epidermis (Wilson and Hemmati-Brivanlou, 1995; Wilson et al., 1997). In ectodermal cells, the alteration of BMP activity using anti-BMP molecules such as noggin or chordin (Zimmerman et al., 1996; Sasai and De Robertis, 1997), truncated type I BMP receptors (Sasai et al., 1995; Suzuki et al., 1997a), BMP4 and BMP7 dominant-negative ligands (Hawley et al., 1995), or antisense *BMP4* RNA (Sasai et al., 1995) block epidermal determination and lead to neural cell fate. Both our in vivo and in vitro analyses showed that Δ*Np63* expression is suppressed when BMP4 is inhibited in *Xenopus*, demonstrating that BMP4 signaling controls Δ*Np63* expression in the ectoderm. These results are in agreement with previous reports that demonstrate the requirement of BMP4 to induce Δ*Np63* expression in ectodermal ES cells (Medawar et al., 2008). In addition, it has been shown that Δ*Np63* is a direct transcriptional target of BMP signaling in zebrafish embryos (Bakkers et al., 2002) and that BMP2-7 are potent inducers of Δ*Np63* expression during mouse tooth and hair development (Laurikkala et al., 2006). All this evidence reinforces the notion that BMP is the upstream signal activating Δ*Np63* to play a key role in controlling epidermal development in vertebrates.

Role of Δ*Np63* During Epidermal Development

Our gain- and loss-of-function experiments showed that Δ*Np63* is required since the earliest steps of epidermal induction in *Xenopus laevis* embryos. The activation of Δ*Np63* led to an expansion of the epidermal territory and suppressed neural markers. Conversely, the blocking of Δ*Np63* functions led to inhibition of the expression of epidermal and cement gland markers and to an ectopic expansion of neural crest and neural plate. Furthermore, our in vitro studies suggest that Δ*Np63* can act as an anti-neuralizing factor in the isolated ectodermal tissue. All these results indicate that Δ*Np63* plays a fundamental role in early epidermal development, mediating the neural versus nonneural fate decision of the ectoderm to become epidermis.

We demonstrated for the first time that the expression of Δ*Np63* overlaps with the placode gene *Six1*, which

suggests that Δ*Np63* could also participate in placodal specification. Previous studies showed that *Six1* overexpression decreases *Sox2* and *FoxD3* expression, whereas the inhibition of *Six1* results in an expansion of the neural plate and neural crest (Brugmann et al., 2004) as we found for Δ*Np63*. Nevertheless, *Six1* represses epidermal genes while Δ*Np63* increases their expression, and the inhibition of *Six1* leads to an expansion of the epidermis which opposes the effect found for Δ*Np63* loss-of-function. Further experiments will be necessary to fully assess the role of Δ*Np63* in placodal development.

Data presented in mice and ES cells are consistent with a role for Δ*Np63* in controlling the proliferation and differentiation of epithelial cells from progenitor precursors in the epidermis (Westfall et al., 2003; Koster et al., 2004; Barbieri et al., 2005; Candi et al., 2006; Truong et al., 2006; Medawar et al., 2008). This evidence supports a role for Δ*Np63* during the process of epidermis differentiation; however, our results demonstrate a very early role for Δ*Np63* in the epidermis induction. The early requirement of Δ*Np63* for a correct development of the epidermis was also shown for zebrafish embryos as well as its participation during epidermal specification. In vivo inhibition of Δ*Np63* by a morpholino oligonucleotide was shown to prevent skin formation, while its exogenous expression promoted epidermal specification (Bakkers et al., 2002; Lee and Kimelman, 2002).

In mammals, Δ*Np63* controls the transcription of genes involved in ectoderm and skin development such as *Dlx3*, *Dlx5*, *Bmp7*, *Notch1*, *Cldn1* (Laurikkala et al., 2006; Radoja et al., 2007; Lo Iacono et al., 2008; Lopardo et al., 2008). Moreover, Δ*Np63* has recently been shown to drive keratinocyte cell fate by directly regulating the basal keratins *K5* and *K14* (Candi et al., 2006; Romano et al., 2007, 2009). In contrast, the expression of many nonepidermal genes is induced by the loss of Δ*Np63* in epidermal cells in the mouse model as well as in human keratinocytes (Barbieri et al., 2006; Truong et al., 2006; De Rosa et al., 2009). Our findings demonstrate that the overexpression and inhibition of Δ*Np63* can modify the expression of epidermal and neural genes. However, we still know very little about Δ*Np63* direct downstream targets in *Xenopus*. The characterization of Δ*Np63* target genes promises to improve our knowledge of the signaling and genetic cascades that are directly involved in epidermal development.

Intriguingly, when Δ*Np63* function was blocked, the expression of the epidermal inner layer marker *Rexp52* showed an expansion. This observation seems to oppose the effect on the epidermal marker *XK81a* that is expressed in the outer layer. A feasible explanation for this could be that Δ*Np63* controls the migration of cell precursors from the inner to the outer ectodermal layer. In the absence of Δ*Np63* these cells probably accumulated in the inner layer as revealed by the *Rexp52* marker expansion. The molecular mechanisms that involve the specification of different cell populations in the inner layer and their passage into the outer layer have recently begun to be understood (Hayes et al., 2007; Dubaissi and Papalopulu, 2011; Quigley et al., 2011), and it will be interesting to evaluate the participation of Δ*Np63* in this process.

ΔNp63 was initially classified as a nontransactivating molecule having a dominant-negative function (Yang et al., 2002). Several experiments in mammal and zebrafish embryos demonstrated that this gene acts as a transcriptional repressor (Bakkers et al., 2002; Westfall et al., 2003; Barton et al., 2009). However, there is evidence supporting the possibility that Δ*Np63* could also act as a transcriptional activator and could regulate the same genes as the TA isoforms possibly by means of a second transactivating domain (Dohn et al., 2001; Ghioni et al., 2002; Wu et al., 2003). In addition to the DNA-binding domain, another common feature of many transcription factors is an additional oligomerization domain (OD). The functional role of oligomerization is to combine the DNA-binding specificity of individual monomeric domains, leading to a substantial increase in binding affinity (Brandt et al., 2009). In our work, we demonstrated that the lack of a Δ*Np63* oligomerization domain generates a dominant negative form of this gene that is able to produce the same phenotypes as Δ*Np63MO*, causing a decreased expression of epidermal gene markers. These results allow us to propose that the OD function is necessary for Δ*Np63* regulation of genes that participate in epidermal fate.

Δ*Np63* Is an Anti-apoptotic Factor During Epidermal Development

During the past few years it has been suggested that Δ*Np63* acts as an anti-apoptotic factor in both cancer and development (Carroll et al., 2006; Cheng et al., 2006; Lee et al., 2006; Ogawa et al., 2008; Borrelli et al., 2009; Mundt et al., 2010). During mammalian development, apoptosis in the bladder epithelium is increased in the absence of Δ*Np63*; however, the restitution of Δ*Np63* expression reverses the increased levels of apoptosis in the urothelium (Cheng et al., 2006). In addition, Δ*Np63* protects human and mouse keratinocytes from apoptosis induced after UVB irradiation (Sanders and Wride, 1995; Liefer et al., 2000; Ogawa et al., 2008). In a breast epithelial cell line, cells expressing Δ*Np63* displayed a reduction in apoptosis after cell detachment (Carroll et al., 2006).

Our studies provide evidence that Δ*Np63* has an anti-apoptotic role during early epidermal development. When Δ*Np63* was overexpressed in vivo, apoptosis inhibition was observed in the injected side of the embryos. Supporting these results, in vitro assays using animal caps or epidermis explants confirmed the anti-apoptotic activity of Δ*Np63*.

The molecular mechanism through which Δ*Np63* inhibits apoptosis was investigated in different models. In hepatocelullar carcinoma cells Δ*Np63* negatively regulates the genes encoding for the death receptor CD95 and the pro-apoptotic Bcl-2 family member *Bax*, interfering with both the death receptor and the mitochondrial apoptosis pathway (Mundt et al., 2010). Furthermore, in urothelial cells the expression of the mitochondrial apoptotic mediators *Bax* and *Apaf1* is increased when Δ*Np63* is inhibited. This effect is rescued by the overexpression of Δ*Np63*, which causes a significantly reduced expression of *Bax*

and *Apaf1* (Cheng et al., 2006). Finally, Δ*Np63* positively regulates the expression of the caspase 8 inhibitor FLIP in both human keratinocytes and mice by controlling the apoptotic pathway (Borrelli et al., 2009).

Our rescue experiments in epidermal explants and in animal caps showed that Δ*Np63* negatively regulates the apoptotic factor *Bax* and lies upstream of this factor in the apoptotic cascade. In addition, Δ*Np63* repressed the transcription of several caspases directly involved in the apoptotic machinery and also increased the expression of the anti-apoptotic factor *Bcl2*. We can speculate that Δ*Np63* leads to the induction of epidermal cells by avoiding their programmed death, thus promoting their survival, specification and differentiation.

Many elements of this model for Δ*Np63* action require additional testing, including the analysis of the functional relationship between Δ*Np63* and other factors known to be important in ectoderm differentiation. Further elucidation of Δ*Np63* regulation could improve our understanding of the molecular events during early embryonic development that support the patterning of the nonneural ectoderm.

In conclusion, we suggest that Δ*Np63* is of extreme importance for the initial induction of *Xenopus* epidermis and participates in the survival-apoptosis balance of this tissue.

EXPERIMENTAL PROCEDURES

Embryonic Manipulation, RNA Microinjection, and Lineage Tracing

Xenopus laevis embryos were obtained by stimulating adult male and female specimens with 400 IU and 800 IU of chorionic gonadotropin (HCG, Elea Lab., Argentina), respectively. Fertilized eggs were obtained after natural single-pair mating and were staged according to the Nieuwkoop and Faber developmental table (1967). Tissue dissections were performed using eyebrow knives as previously described (Tribulo et al., 2004; Bonano et al., 2008). RNA microinjection and lineage tracing were carried out as described previously (Aybar et al., 2003; Tribulo et al., 2003).

Morpholino Antisense Oligonucleotide and DNA Constructs

A morpholino antisense oligonucleotide (Δ*Np63MO*) was synthesized against *Xenopus laevis* Δ*Np63* from the initiation start site with the following sequence 5′-AGCACTGTTTTCCA GATACAACATC-3′ (Gene Tools, LLC). A morpholino corresponding to the same sequence was previously reported and its action was initially characterized by Barton et al. (2009). A 40 ng/embryo dose was microinjected together with a lineage tracer into one blastomere at the 8- or 16-cell stage. A control antisense oligonucleotide composed of a random sequence (*CoMO*, Gene Tools, LLC) was injected as a control. To test the efficacy of Δ*Np63MO* and also to have another tool to inhibit the function of Δ*Np63*, a C-terminal truncated form of Δ*Np63* fused to GFP, termed Δ*Np63(Ct)-GFP*, was generated as follows. High-fidelity PCR was performed using as a template *pCS64T-*Δ*Np63* and the following primers: 5′-GGATCCATGTTGTATCTGGAAAAC AATGCTC-3′ (underlined *Bam*HI restriction site) and 5′-CCATGGC-TGAAACTTG-CTGTTTTCTGATGC-3′ (underlined *Nco*I restriction site). An 814-bp fragment encoding only the ΔN- N-terminal region and the DNA binding domain (DBD) of Δ*Np63* was amplified and cloned into the TOPO-TA vector. Then, this fragment was directionally cloned into the *Bam*HI and *Nco*I sites of the *pCS2-EGFP* vector to produce the Δ*Np63(Ct)-GFP* construct, which lacks the 284-bp coding for the oligomerization domain (OD) (Brandt et al., 2009). In vitro transcribed mRNA of this construct was injected alone or with the morpholino oligonucleotide. *CM-BMP4* and *dnBMPR* constructs were donated by Dr. K.W. Cho (Hawley et al., 1995; Blitz et al., 2000) and Δ*Np63* was provided by Dr. P. Vize (Lu et al., 2001). All cDNAs were linearized and transcribed as described previously (Tribulo et al., 2004).

Western Blotting

Embryos were homogenized in the extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% NP-40 and a protease inhibitor cocktail containing 2 mM PMSF, 0.5 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 μg/ml aprotinin). Protein determination was performed by the method of Lowry et al. (1951). Samples of 100 μg were loaded and separated in a 7.5% Tris-HCl sodium dodecyl sulfate-polyacrylamide electrophoresis gel and blotted to a nitrocellulose membrane. The membranes were blocked and incubated with polyclonal anti-GFP antibody (Invitrogen) primarily and then with a secondary antibody associated with Peroxidase (Sigma-Aldrich) and developed with the substrate (Aybar et al., 1996).

RNA Isolation From Embryos and RT-PCR Analysis

Total RNA was isolated from embryonic tissues after microdissection and cDNAs were synthesized as previously described (Aybar et al., 2003). The primers designed for this study were: Δ*Np63*, 5′-ATGTTGTATCTGGA AAACAATGCTCAG-3′ and 5′-GACAA CGCTTCACAACCTCTG-3′ (these primers enable the specific detection of Δ*Np63* since they are targeted to the 5′ region of the cDNA); *XK81a*, 5′-CACCAGAACACAGAGTAC-3′ and 5′-CAACCTTCCATCAACCA-3′; *Dlx3*, 5′-ACAATAGTGGAAGCCGAACC-3′ and 5′-AACCATTTGAGGGATCAAGC-3′; *Sox2*, 5-GAGGATGGACACTTATGCC CAC-3′ and 5′-GGACATGCTGTAGG TAGGCGA-3′; *XBra*, 5′-GCTGGAAG TATGTGAATGGAG-3′ and 5′-TTAA GTGCTGTAATCTCTTCA-3′; *Caspase 3*, 5′-AAGTCTGGAACATCGCAAGG-3′ and 5′-TAAATGAGCCCCTCATCACC-3′; *Caspase 9*, 5′-CGGGAAAGAATTG GAAGTCA-3′ and 5′-AGGGCCGTTCT GTAAACCTT-3′; *Bcl2*, 5′-GTCGGCCT GTATGGAAAGAA-3′ and 5′-CATGAT AGGCGACCCAGTG-3′, *Bax*, 5′-GCCT GAGGAAAATAGGTGATGAGC-3′ and 5′-GGACATCTTCCAGATGGCAAGAG; *EF1*α, 5′-CAGATTGGTGCTGGATAT GC-3′ and 5′-ACTGCCTTGATGACTC CTAG-3′. PCR amplification with these primers was performed over 29 cycles and the PCR products were analyzed on 1.5% agarose gels. PCR on RNA that had not been reverse-transcribed was performed to check for DNA contamination. Quantitation of PCR bands was performed using ImageJ software (NIH, USA) on

eight-bit grayscale JPG files and the values were normalized to the *EF1α* levels from the same sample and expressed for comparison as relative intensities (sample/*EF1α*X10).

In Situ Hybridization and TUNEL

Antisense probes containing digoxigenin-11-UTP or fluorescein-12-UTP were prepared for Δ*Np63* (Lu et al., 2001), *Sox2* (Dr. R.M. Grainger, personal communication), cytokeratin *XK81a* (Jonas et al., 1985), *FoxD3* (Sasai et al., 2001), *Dlx3* and *Dlx5* (Luo et al., 2001), *Six1* (Pandur and Moody, 2000), and *Rexp52* (Pollet et al., 2005) by in vitro transcription. Specimens were prepared, hybridized and stained as previously described (Aybar et al., 2003; Tribulo et al., 2003). Apoptosis was detected by TUNEL staining according to the procedure previously described (Hensey and Gautier, 1998; Tribulo et al., 2004; Bonano et al., 2008).

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REFERENCES

Aybar MJ, Genta SB, Villecco EI, Riera AN, Sanchez SS. 1996. Amphibian Bufo arenarum vitronectin-like protein: its localization during oogenesis. Int J Dev Biol 40:997–1008.

Aybar MJ, Nieto MA, Mayor R. 2003. Snail precedes slug in the genetic cascade required for the specification and migration of the Xenopus neural crest. Development 130:483–494.

Baehrecke EH. 2002. How death shapes life during development. Nat Rev Mol Cell Biol 3:779–787.

Bakkers J, Hild M, Kramer C, Furutani-Seiki M, Hammerschmidt M. 2002. Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. Dev Cell 2:617–627.

Barbieri CE, Perez CA, Johnson KN, Ely KA, Billheimer D, Pietenpol JA. 2005. IGFBP-3 is a direct target of transcriptional regulation by DeltaNp63alpha in squamous epithelium. Cancer Res 65: 2314–2320.

Barbieri CE, Tang LJ, Brown KA, Pietenpol JA. 2006. Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. Cancer Res 66: 7589–7597.

Barton CE, Tahinci E, Barbieri CE, Johnson KN, Hanson AJ, Jernigan KK, Chen TW, Lee E, Pietenpol JA. 2009. DeltaNp63 antagonizes p53 to regulate mesoderm induction in Xenopus laevis. Dev Biol 329:130–139.

Blitz IL, Shimmi O, Wunnenberg-Stapleton K, O'Connor MB, Cho KW. 2000. Is chordin a long-range- or short-range-acting factor? Roles for BMP1-related metalloproteases in chordin and BMP4 autofeedback loop regulation. Dev Biol 223:120–138.

Bonano M, Tribulo C, De Calisto J, Marchant L, Sanchez SS, Mayor R, Aybar MJ. 2008. A new role for the Endothelin-1/Endothelin-A receptor signaling during early neural crest specification. Dev Biol 323:114–129.

Borrelli S, Candi E, Alotto D, Castagnoli C, Melino G, Vigano MA, Mantovani R. 2009. p63 regulates the caspase-8-FLIP apoptotic pathway in epidermis. Cell Death Differ 16:253–263.

Brandt T, Petrovich M, Joerger AC, Veprintsev DB. 2009. Conservation of DNA-binding specificity and oligomerisation properties within the p53 family. BMC Genomics 10:628.

Brugmann SA, Pandur PD, Kenyon KL, Pignoni F, Moody SA. 2004. Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. Development 131:5871–5881.

Candi E, Rufini A, Terrinoni A, Dinsdale D, Ranalli M, Paradisi A, De Laurenzi V, Spagnoli LG, Catani MV, Ramadan S, Knight RA, Melino G. 2006. Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. Cell Death Differ 13:1037–1047.

Carroll DK, Carroll JS, Leong CO, Cheng F, Brown M, Mills AA, Brugge JS, Ellisen LW. 2006. p63 regulates an adhesion programme and cell survival in epithelial cells. Nat Cell Biol 8:551–561.

Chalmers AD, Lachani K, Shin Y, Sherwood V, Cho KW, Papalopulu N. 2006. Grainyhead-like 3, a transcription factor identified in a microarray screen, promotes the specification of the superficial layer of the embryonic epidermis. Mech Dev 123:702–718.

Cheng W, Jacobs WB, Zhang JJ, Moro A, Park JH, Kushida M, Qiu W, Mills AA, Kim PC. 2006. DeltaNp63 plays an anti-apoptotic role in ventral bladder development. Development 133:4783–4792.

De Rosa L, Antonini D, Ferone G, Russo MT, Yu PB, Han R, Missero C. 2009. p63 Suppresses non-epidermal lineage markers in a bone morphogenetic protein-dependent manner via repression of Smad7. J Biol Chem 284: 30574–30582.

Dohn M, Zhang S, Chen X. 2001. p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. Oncogene 20:3193–3205.

Dubaissi E, Papalopulu N. 2011. Embryonic frog epidermis: a model for the study of cell-cell interactions in the development of mucociliary disease. Dis Model Mech 4:179–192.

Ghioni P, Bolognese F, Duijf PH, Van Bokhoven H, Mantovani R, Guerrini L. 2002. Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains. Mol Cell Biol 22:8659–8668.

Hayes JM, Kim SK, Abitua PB, Park TJ, Herrington ER, Kitayama A, Grow MW, Ueno N, Wallingford JB. 2007. Identification of novel ciliogenesis factors using a new in vivo model for mucociliary epithelial development. Dev Biol 312:115–130.

Hawley SH, Wunnenberg-Stapleton K, Hashimoto C, Laurent MN, Watabe T, Blumberg BW, Cho KW. 1995. Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. Genes Dev 9:2923–2935.

Hensey C, Gautier J. 1998. Programmed cell death during Xenopus development: a spatio-temporal analysis. Dev Biol 203:36–48.

Jonas E, Sargent TD, Dawid IB. 1985. Epidermal keratin gene expressed in embryos of Xenopus laevis. Proc Natl Acad Sci U S A 82:5413–5417.

King KE, Ponnamperuma RM, Gerdes MJ, Tokino T, Yamashita T, Baker CC, Weinberg WC. 2006. Unique domain functions of p63 isotypes that differentially regulate distinct aspects of epidermal homeostasis. Carcinogenesis 27:53–63.

Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M, Roop DR. 2007. p63 induces key target genes required for epidermal morphogenesis. Proc Natl Acad Sci U S A 104:3255–3260.

Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR. 2004. p63 is the molecular switch for initiation of an epithelial stratification program. Genes & development 18:126–131.

Koster MI, Roop DR. 2004. Genetic pathways required for epidermal morphogenesis. Eur J Cell Biol 83:625–629.

LaBonne C, Bronner-Fraser M. 1998. Neural crest induction in Xenopus: evidence for a two-signal model. Development 125:2403–2414.

Laurikkala J, Mikkola ML, James M, Tummers M, Mills AA, Thesleff I. 2006. p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. Development 133:1553–1563.

Lee H, Kimelman D. 2002. A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev Cell 2:607–616.

Lee HO, Lee JH, Choi E, Seol JY, Yun Y, Lee H. 2006. A dominant negative form of p63 inhibits apoptosis in a p53-independent manner. Biochem Biophys Res Commun 344:166–172.

Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F, Roop DR. 2000. Down-regulation of p63 is required for epidermal

UV-B-induced apoptosis. Cancer Res 60: 4016–4020.
Lo Iacono N, Mantero S, Chiarelli A, Garcia E, Mills AA, Morasso MI, Costanzo A, Levi G, Guerrini L, Merlo GR. 2008. Regulation of Dlx5 and Dlx6 gene expression by p63 is involved in EEC and SHFM congenital limb defects. Development 135:1377–1388.
Lopardo T, Lo Iacono N, Marinari B, Giustizieri ML, Cyr DG, Merlo G, Crosti F, Costanzo A, Guerrini L. 2008. Claudin-1 is a p63 target gene with a crucial role in epithelial development. PloS one 3:e2715.
Lu P, Barad M, Vize PD. 2001. Xenopus p63 expression in early ectoderm and neurectoderm. Mech Dev 102:275–278.
Luo T, Matsuo-Takasaki M, Lim JH, Sargent TD. 2001. Differential regulation of Dlx gene expression by a BMP morphogenetic gradient. Int J Dev Biol 45:681–684.
Luo T, Matsuo-Takasaki M, Thomas ML, Weeks DL, Sargent TD. 2002. Transcription factor AP-2 is an essential and direct regulator of epidermal development in Xenopus. Dev Biol 245:136–144.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. J Biol Chem 193:265–275.
Ma CM. 2009. Nkx-2.3 gene in mouse epidermal maturation. Biol Res 42:267–280.
Marchant L, Linker C, Ruiz P, Guerrero N, Mayor R. 1998. The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. Dev Biol 198:319–329.
Medawar A, Virolle T, Rostagno P, de la Forest-Divonne S, Gambaro K, Rouleau M, Aberdam D. 2008. DeltaNp63 is essential for epidermal commitment of embryonic stem cells. PloS One 3:e3441.
Mikkola ML. 2007. p63 in skin appendage development. Cell Cycle 6:285–290.
Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398:708–713.
Mundt HM, Stremmel W, Melino G, Krammer PH, Schilling T, Muller M. 2010. Dominant negative (DeltaN) p63alpha induces drug resistance in hepatocellular carcinoma by interference with apoptosis signaling pathways. Biochem Biophys Res Commun 396:335–341.
Nieuwkoop PD, Faber J. 1967. Normal table of Xenopus laevis (Daudin). Amsterdam: North Holland.
Ogawa E, Okuyama R, Ikawa S, Nagoshi H, Egawa T, Kurihara A, Yabuki M, Tagami H, Obinata M, Aiba S. 2008. p51/p63 Inhibits ultraviolet B-induced apoptosis via Akt activation. Oncogene 27:848–856.
Onichtchouk D, Gawantka V, Dosch R, Delius H, Hirschfeld K, Blumenstock C, Niehrs C. 1996. The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controling] dorsoventral patterning of Xenopus mesoderm. Development 122: 3045–3053.
Pandur PD, Moody SA. 2000. Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines. Mech Dev 96:253–257.
Patturajan M, Nomoto S, Sommer M, Fomenkov A, Hibi K, Zangen R, Poliak N, Califano J, Trink B, Ratovitski E, Sidransky D. 2002. DeltaNp63 induces beta-catenin nuclear accumulation and signaling. Cancer Cell 1:369–379.
Pollet N, Muncke N, Verbeek B, Li Y, Fenger U, Delius H, Niehrs C. 2005. An atlas of differential gene expression during early Xenopus embryogenesis. Mech Dev 122:365–439.
Quigley IK, Stubbs JL, Kintner C. 2011. Specification of ion transport cells in the Xenopus larval skin. Development 138:705–714.
Radoja N, Guerrini L, Lo Iacono N, Merlo GR, Costanzo A, Weinberg WC, La Mantia G, Calabro V, Morasso MI. 2007. Homeobox gene Dlx3 is regulated by p63 during ectoderm development: relevance in the pathogenesis of ectodermal dysplasias. Development 134: 13–18.
Romano RA, Birkaya B, Sinha S. 2007. A functional enhancer of keratin14 is a direct transcriptional target of deltaNp63. J Invest Dermatol 127:1175–1186.
Romano RA, Ortt K, Birkaya B, Smalley K, Sinha S. 2009. An active role of the DeltaN isoform of p63 in regulating basal keratin genes K5 and K14 and directing epidermal cell fate. PloS One 4:e5623.
Sanders EJ, Wride MA. 1995. Programmed cell death in development. Int Rev Cytol 163:105–173.
Sasai N, Mizuseki K, Sasai Y. 2001. Requirement of FoxD3-class signaling for neural crest determination in Xenopus. Development 128:2525–2536.
Sasai Y, De Robertis EM. 1997. Ectodermal patterning in vertebrate embryos. Dev Biol 182:5–20.
Sasai Y, Lu B, Steinbeisser H, De Robertis EM. 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature 376:333–336.
Schlosser G, Ahrens K. 2004. Molecular anatomy of placode development in Xenopus laevis. Dev Biol 271:439–466.
Senoo M, Pinto F, Crum CP, McKeon F. 2007. p63 Is essential for the proliferative potential of stem cells in stratified epithelia. Cell 129:523–536.
Suzuki A, Kaneko E, Ueno N, Hemmati-Brivanlou A. 1997a. Regulation of epidermal induction by BMP2 and BMP7 signaling. Dev Biol 189:112–122.
Suzuki A, Ueno N, Hemmati-Brivanlou A. 1997b. Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. Development 124:3037–3044.
Tao J, Kuliyev E, Wang X, Li X, Wilanowski T, Jane SM, Mead PE, Cunningham JM. 2005. BMP4-dependent expression of Xenopus Grainyhead-like 1 is essential for epidermal differentiation. Development 132:1021–1034.
Tomimori Y, Katoh I, Kurata S, Okuyama T, Kamiyama R, Ikawa Y. 2004. Evolutionarily conserved expression pattern and trans-regulating activity of Xenopus p51/p63. Biochem Biophys Res Commun 313:230–236.
Tribulo C, Aybar MJ, Nguyen VH, Mullins MC, Mayor R. 2003. Regulation of Msx genes by a Bmp gradient is essential for neural crest specification. Development 130:6441–6452.
Tribulo C, Aybar MJ, Sanchez SS, Mayor R. 2004. A balance between the anti-apoptotic activity of Slug and the apoptotic activity of msx1 is required for the proper development of the neural crest. Dev Biol 275:325–342.
Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA. 2006. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev 20:3185–3197.
Westfall MD, Mays DJ, Sniezek JC, Pietenpol JA. 2003. The Delta Np63 alpha phosphoprotein binds the p21 and 14–3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. Mol Cell Biol 23: 2264–2276.
Wilson PA, Hemmati-Brivanlou A. 1995. Induction of epidermis and inhibition of neural fate by Bmp-4. Nature 376: 331–333.
Wilson PA, Lagna G, Suzuki A, Hemmati-Brivanlou A. 1997. Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. Development 124: 3177–3184.
Wu G, Nomoto S, Hoque MO, Dracheva T, Osada M, Lee CC, Dong SM, Guo Z, Benoit N, Cohen Y, Rechthand P, Califano J, Moon CS, Ratovitski E, Jen J, Sidransky D, Trink B. 2003. DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development. Cancer Res 63:2351–2357.
Yang A, Kaghad M, Caput D, McKeon F. 2002. On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet 18:90–95.
Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F. 1998. p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 2:305–316.
Yasue A, Tao H, Nohno T, Moriyama K, Noji S, Ohuchi H. 2001. Cloning and expression of the chick p63 gene. Mech Dev 100:105–108.
Zimmerman LB, De Jesus-Escobar JM, Harland RM. 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86:599–606.