Development 133, 4783-4792 (2006) doi:10.1242/dev.02621

ΔNp63 plays an anti-apoptotic role in ventral bladder development

Wei Cheng[1,2], W. Bradley Jacobs[3], Jennifer J. R. Zhang[1,2], Anne Moro[1,2], Jin-Hyung Park[1,2], Michelle Kushida[1,2], Wei Qiu[2], Alea A. Mills[4] and Peter C. W. Kim[1,2,*]

The bladder, the largest smooth-muscle organ in the human body, is responsible for urine storage and micturition. *P63,* a homolog of the *p53* tumor-suppressor gene, is essential for the development of all stratified epithelia, including the bladder urothelium. The N-terminal truncated isoform of *p63*, *ΔNp63*, is known to have anti-apoptotic characteristics. We have established that *ΔNp63* is not only the predominant isoform expressed throughout the bladder, but is also preferentially expressed in the ventral bladder urothelium during early development. We observed a host of ventral defects in $p63^{-/-}$ embryos, including the absence of the abdominal and ventral bladder walls. This number of ventral defects is identical to bladder exstrophy, a congenital anomaly exhibited in human neonates. In the absence of *p63*, the ventral urothelium was neither committed nor differentiated, whereas the dorsal urothelium was both committed and differentiated. Furthermore, in $p63^{-/-}$ bladders, apoptosis in the ventral urothelium was significantly increased. This was accompanied by the upregulation of mitochondrial apoptotic mediators *Bax* and *Apaf1*, and concurrent upregulation of *p53*. Overexpression of *ΔNp63γ* and *ΔNp63β* in $p63^{-/-}$ bladder primary cell cultures resulted in a rescue, evidenced by significantly reduced expressions of *Bax* and *Apaf1*. We conclude that *ΔNp63* plays a crucial anti-apoptotic role in normal bladder development.

KEY WORDS: *p63*, Bladder exstrophy, Apoptosis, Mouse

INTRODUCTION

Bladder exstrophy (BE) is a serious congenital anomaly affecting one in 36,000 live births (Martinez-Frias et al., 2001). In cases of BE, the ventral abdominal and bladder walls are either absent, leaving the bladder cavity exposed, or covered only by an amniotic sac; the pubic bones, external genitalia and rectus abdominis muscles are separated along the midline, whereas the anus is displaced ventrally, often with an associated narrowing or atresia. Treatment of BE is complicated and involves major reconstructive surgical procedures. Although advanced parental age (Boyadjiev et al., 2004), familial links (Shapiro et al., 1984) and racial predilection (Roberts et al., 1995) imply a genetic cause, the molecular mechanisms underlying the formation of BE remain unknown. As such, our understanding of the pathogenesis of BE remains limited to that provided by a previous descriptive study (Muecke, 1964).

During embryogenesis, the cloacal cavity at the posterior end of the embryo is partitioned by the uro-rectal septum into the ventral urogenital sinus (UGS) and the dorsal hindgut. The UGS subsequently develops into the urethra, bladder and urachus. The UGS epithelium differentiates into a stratified transitional epithelium, known as the urothelium, whereas the mesenchyme of the UGS differentiates into the lamina propria and the smooth muscle of the bladder, known as the detrusor muscle. Interaction between the UGS epithelium and its mesenchyme is crucial for proper development of the detrusor muscle, as previous studies have shown that the UGS epithelium provides key signaling input that promotes differentiation of the UGS mesenchyme into smooth muscle (Baskin et al., 1996b).

Homologs *p53*, *p63* and *p73* comprise the p53 gene family (Levine, 1997; Murray-Zmijewski et al., 2006). *p63* is highly expressed in all stratified epithelia and its expression can be detected in the urothelium as early as E11.5 and persisting thereafter (Kurita and Cunha, 2001; Kurita et al., 2004a; Kurita et al., 2004b). We therefore hypothesize that *p63* plays a role in bladder urothelium development that, in turn, affects bladder development. $p63^{-/-}$ mice exhibit severe developmental anomalies, including failure of skin morphogenesis, truncation of limbs and craniofacial abnormalities (Mills et al., 1999; Yang et al., 1999). The specific mechanism underlying the regulation of epithelial stratification and development by *p63* is not fully delineated and remains controversial. Some investigators suggest that failure of epithelial stratification in the absence of *p63* is related to a lack of commitment (Koster et al., 2004; Mills et al., 1999), whereas others suggest that it results from a defect in epithelial cell proliferation (McKeon, 2004; Yang et al., 1999).

p63 expresses multiple N-terminal isoforms, known as *TAp63* and *ΔNp63*, because of the presence of an alternative promoter located in intron 3. The full-length isoform, *TAp63*, contains a transactivation (TA) domain similar to the TA domain of *p53*. *TAp63* is capable of activating numerous *p53* target genes, promoting cell-cycle arrest (Yang et al., 1999) and inducing apoptosis (Jacobs et al., 2005). Conversely, the truncated isoform, *ΔNp63*, acts in a dominant-negative manner towards the TA isoforms of p63 and p53 (Yang et al., 1998). *ΔNp63* has been shown to inhibit apoptosis (Jacobs et al., 2005) and to promote stem-cell proliferation in vitro (Moll and Slade, 2004). In addition to these N-terminal isoforms, alternative splicing at the C-terminus of *p63* generates three isoforms: α, β and γ. In combination with the N-terminal isoforms, six *p63* isoforms can be generated (Yang et al., 1998).

[1]Department of Surgery, [2]Program for Infection, Immunity, Injury and Repair and [3]Program of Developmental Biology, Hospital for Sick Children, Toronto, M5G 1X8, Canada. [4]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

*Author for correspondence (e-mail: peter.kim@sickkids.ca)

Accepted 8 September 2006

In the current study, we find that the *ΔNp63* isoform is the predominant isoform in the ventral bladder throughout development. In the absence of *p63*, the abdominal and ventral bladder walls are absent; these defects epitomize the BE complex in humans. In addition, the ventral epithelium of the *p63*$^{-/-}$ bladder is neither committed to stratification nor differentiated, and exhibits significantly increased apoptotic activity. Pro-apoptotic mediators *Bax* and *Apaf1* are upregulated in the *p63*$^{-/-}$ bladder. Restoration of *ΔNp63β* or *ΔNp63γ* protein levels in *p63*$^{-/-}$ bladders partially rescues expression of *Bax* and *Apaf1*. Furthermore, absence of *p63* in the bladder epithelium leads to failure of induction of the adjacent UGS mesenchyme, resulting in a significant reduction of mesenchymal proliferation. Taken together, these observations lead us to conclude that *ΔNp63* plays a crucial anti-apoptotic role in the development of the ventral bladder epithelium.

MATERIALS AND METHODS

p63$^{-/-}$ mutant mice genotyping

p63$^{-/-}$ mutant mice were bred on a C57B16 background (Mills et al., 1999). Homozygous embryos were identified by phenotype. Heterozygous embryos were genotyped by PCR (primers 5′-GTGTTGGCAAGGATTCTGAGACC-3′ and 5′-GGAAGACAATAGCAGGCATGCTG-3′).

Histochemistry and immunohistochemistry

Specimen sections (7 μm) were stained with 50% hematoxylin and 0.5% Eosin in 70% ethanol. Carbohydrates were stained with periodic acid and Schiff reaction (PAS, Surgipath). Alkaline phosphatase (AP) reaction was studied by treating slides with 0.1 M Tris-buffered solution (pH 9.5) followed by the addition of BM purple AP substrate (Roche).

Immunochemistry was performed as follows: after quenching the endogenous peroxidases with 3% H_2O_2 in 10% methanol, the antigens were retrieved by boiling the slides in an antigen-unmasking solution (H-3300, Vector Laboratories). The sections were blocked with blocking reagent (Roche). Primary antibodies at the following dilutions were applied: cytokeratin 18 (CK18) (1:100, Santa Cruz Biotechnology), *p63* (4A4, 1:100, Santa Cruz Biotechnology), *TAp63* (1:20, Santa Cruz Biotechnology), *ΔNp63* (1:100, gift from Dr K Nylander) (Nylander et al., 2002), *p53* (1:250, Abcam, ab26), *p73* (1:200, Abcam, ab17230), villin (1:100, Santa Cruz Biotechnology), uroplakin 3 (undiluted, Santa Cruz Biotechnology), smooth-muscle α-actin (undiluted, Sigma Chemicals), cleaved caspase-3 (1:100, Sigma Chemicals), Msx1 (1:500, Covance Research Products) and smooth-muscle heavy-chain myosin (1: 2000, Santa Cruz Biotechnology). Appropriate secondary antibodies were applied at 1:200 dilutions. Avidin-biotin-peroxidase complex (ABC)-buffer washing was followed by substrate diaminobenzidine (DAB) staining. Cell proliferation was assayed by 5-bromo-2′deoxyuridine (BrdU) incorporation (animals were injected with 100 μm BrdU per gram of bodyweight). Apoptosis was studied using the terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) assay and FragEL DNA Fragmentation Detection Kit (Calbiochem).

RNA extraction, qPCR and RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the SuperScript II First-Strand Synthesis Kit (Invitrogen), then purified using a QIAquick PCR Purification Kit (Qiagen). Quantitative polymerase chain reactions (qPCRs) were performed using commercially available *p53* primers (SuperArray Bioscience, PPM02931A-24) and self-designed primers: *p53* (5′-CACCTCACTGCATGGACGATC-3′, 5′-GTCTGCCTGTCTTCCAGATACTCG-3′, T: 59.1°C); *p73* (5′-CAAGAAGGCAGAGCATGTGA-3′, 5′-TCATACGGCACAACCACACT-3′, T: 50.1°C); *β-actin* (internal control, 5′-CCTTTTCCAGCCTTCCTTC-3′, 5′-TACTCCTGCTTGCTGATCC-3′, T: 55.0°C); *Bax* (5′-CGAGCTGATCAGAACCATCA-3′, 5′-CTCAGCCCATCTTCTTCCAG-3′, T: 50.1°C); and *Apaf1* (5′-GAGAAAACCCTGAGGCACAA-3′, 5′-TAATTAAAGCGGCTGCTCGT-3′, T: 50.4°C). The relative expressions were analyzed according to Pfaffl's methods (Pfaffl, 2001).

Reverse transcriptase-polymerase chain reactions (RT-PCRs) were performed using the following primers: *ΔNp63* (5′-CAATGCCCAGACTCAATTTAGTGA-3′, 5′-GGCCCGGGTAATCTGTGTTGG-3′, 221bp, T: 51.4°C); *TAp63* (5′-AACCCCAGCTCATTTCTCTG-3′, 5′-GGCCCGGGTAATCTGTGTTGG-3′, 449 bp, T: 57.0°C); *p63α* (5′-ACGGGGTGGAAAAGAGATGGTC-3′, 5′-AAGAGACCGGAAGGCAGATGAAG-3′, 919 bp, T: 59.5°C); *p63β* (5′-GACTTGCCAAATCCTGACA-3′, 5′-AAGAGACCGGAAGGCAGATGAAG-3′, 619 bp, 55.1°C); and *p63γ* (5′-CTCCCCGGGGCTCCACAAG-3′, 5′-AAGAGACCGGAAGGCAGATGAAG-3′, 338 bp, T: 56.2°C).

Immunoblot

Immunoblot was performed as previously described (Qiu et al., 2004). Briefly, the cultured cells were washed twice with 1×PBS and lysed using a solubilizing buffer (1×PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 100 KIU/mL Trasylol and 0.5 μM ALLN), and an equal amount of cell lysates were resolved on 8% SDS-PAGE mini gels. Following SDS-PAGE, the protein was transferred electrophoretically for 18 hours at 4°C onto PVDF. The membranes were blocked with a 4% solution of fat-free dry milk powder, incubated with the primary antibodies (anti-Bax antibody, Upstate Cell Signaling, cat. no. 06-499, 1:500; anti-*Apaf1*, Chemicon, cat. no. MAB3504, 1:500; and anti-β-actin antibody, Sigma Chemicals, cat. no. A 5441, 1:1000), washed, and incubated with a secondary antibody conjugated to horseradish peroxidase. Membranes were then incubated in an enhanced chemiluminescence-detection reagent (Amersham Pharmacia Biotech) and exposed to Kodak Hyperfilm (Eastman Kodak). Films were developed and quantitative analysis was performed using an imaging densitometer.

Organ culture, primary cell culture and transfection

The dissected bladders were cultured in 50% BGJb medium (Invitrogen), plus 50% A10 (Wisent) culture medium with supplements of transferrin (20 ng/ml), insulin (10 ng/ml) and epithelial growth factor (10 ng/ml). Primary cells were cultured with Eagle's minimum essential medium (EMEM; Wisent) and 20% fresh bovine serum (FBS). Green fluorescent protein (GFP)-tagged recombinant bicistronic adenoviruses with *ΔNp63γ, ΔNp63β*, and *TAp63γ* constructs were generated, purified and titered as previously reported (Jacobs et al., 2005). The adenoviruses were added to the culture media at the ratio of 5-10 pfu per cell.

In situ hybridization

In situ hybridization of paraffin sections with a DIG-labeled RNA probe was performed as previously described (Hui and Joyner, 1993). Briefly, the dewaxed slides were pre-fixed with 4% paraformaldehyde, permeabilized with proteinase K (0.02 mg/ml), and treated with 0.2 M HCl solution and 0.1 M triethanoloamine solution (TEA), plus 0.025 ml acetic anhydride/liter of TEA. The slides were then hybridized with 4.0 μg/ml of DIG-labeled RNA probes (DIG labeling mix; Roche) in a formamide/sodium citrate-sodium chloride (SSC) buffer in a 55°C oven overnight. The slides were then washed with a 5×SSC/formamide solution. and treated with RNAse-A, 2×SSC and 0.2×SSC before being blocked with blocking reagent (Roche). Anti-DIG alkaline phosphatase antibody (Roche) was then applied, followed by BM purple AP substrate (Roche).

RESULTS

p63 deficiency leads to bladder exstrophy

In humans, BE complex is evidenced by a cluster of ventral midline defects, including: (1) ventral abdominal- and ventral bladder-wall defects; (2) bifid external genitalia; and (3) separation of the pubic bones (Fig. 1A,B). All *p63*$^{-/-}$embryos examined (*n*=12) developed bladder abnormalities. Four embryos developed BE with ventral bladder- and abdominal-wall defects (with and without membrane cover), bifid external genitalia (Fig. 1C,D) and umbilical hernia. The remaining eight embryos developed dilated bladders with both thin lamina propria and thin muscle layers. The sagittal sections of E18.5 *p63*$^{-/-}$-mutant embryos demonstrated the full complement of BE (Fig. 1E,F), as evidenced by: (1) ventral abdominal-wall defect; (2)

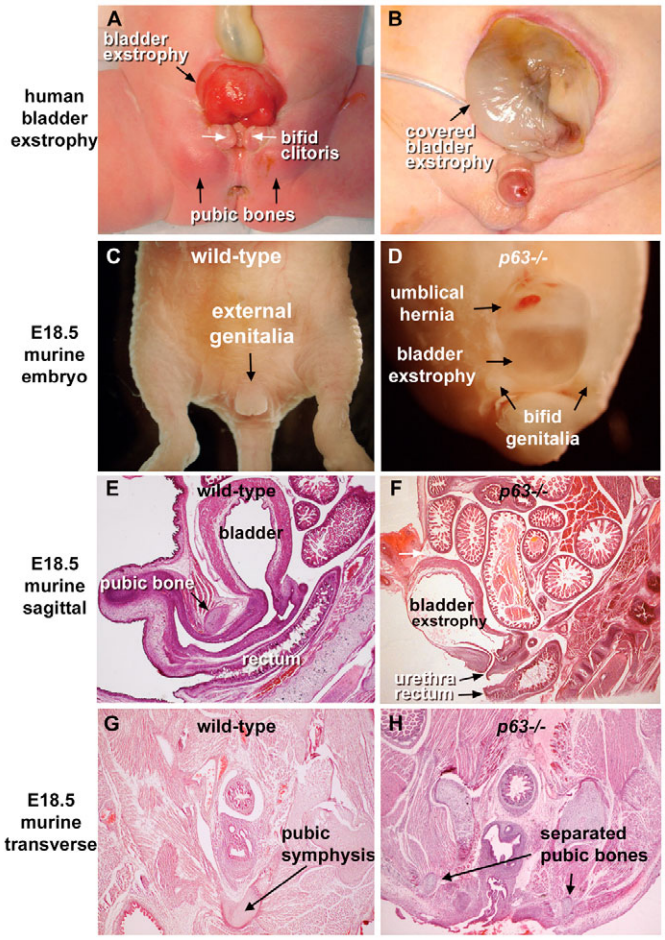

Fig. 1. BE in humans and *p63*$^{-/-}$ mice. (**A**) BE with separation of pubic bones and genitalia in a female. (**B**) Covered BE in a male. (**C**) The wild-type E18.5 embryo (10×). (**D**) BE in an E18.5 *p63*$^{-/-}$ embryo (10×). (**E**,**F**) Hematoxylin and Eosin staining of sagittal sections of wild-type (E) and *p63*$^{-/-}$ (F) E18.5 embryos (40×). White arrow: umbilical hernia. (**G**,**H**) Hematoxylin and Eosin staining of transverse sections of E18.5 wild-type (G) and *p63*$^{-/-}$ (H) embryo pelvises (40×). (Image A,B courtesy of J. L. Salle, Hospital for Sick Children, Toronto, Canada).

ventral bladder-wall defect covered with a thin membrane; (3) absence of pubic symphysis at the midline (i.e. separation of the pubic bones); (4) absence of external genitalia at the midline (i.e. bifid genitalia); (5) umbilical hernia; and (6) ventral translocation of the anus. Sections of even younger embryos demonstrated that the separation of external genitalia was evident at E11.5 (Fig. 5A,B). Transverse sections through the *p63*$^{-/-}$-embryo pelvis confirmed BE (ruptured membrane and separation of pubic bones; Fig. 1G,H). In summary, the *p63*$^{-/-}$ mutant mouse phenotype recapitulates the full spectrum of human BE complex.

p63 is expressed in bladder epithelium throughout its development and ΔNp63 is the predominant isoform

To define the role of *p63* in bladder development, the ontogeny of *p63* expression in the bladder was examined by immunohistochemistry from gestational days E11.5 to E17.5 using a 4A4 pan-*p63* antibody (Fig. 2A-D). *p63* was initially expressed in the distal-ventral UGS epithelium, hindgut and skin overlying the genital tubercle of E11.5-E12.5 embryos (Fig. 2A,B). *p63* expression then extended to the epithelium over the body and dome of the bladder at E15.5 and E17.5, respectively (Fig. 2C,D). Next, the expression pattern of the different N-terminal isoforms was studied using antibodies specific to either *TAp63* or *ΔNp63* (Nylander et al., 2002). Expression of the *ΔNp63* isoform (detected by anti-*ΔNp63* antibody) was found to be similar to that of the pan-*p63* antibody (detected by 4A4), suggesting that *ΔNp63* represents the predominant isoform during bladder development. *ΔNp63* expression began in the ventral UGS epithelium at E11.5 and extended to the rest of the epithelium later in development (Fig. 2E-H). *ΔNp63* was also expressed in the epithelium overlying the urogenital tubercle (Fig. 2E,F). By contrast, *TAp63* (detected by anti-*TAp63*) was expressed only transiently from E11.5 to E12.5 in the epithelium of the distal hindgut and in its communication with the UGS (Fig. 2I,J). *TAp63* expression decreased markedly in the distal hindgut after E14.5 (Fig. 2K,L and data not shown) and was not expressed in the skin overlying the urogenital tubercle.

To verify the dominant *p63* N-terminal isoform expressed in wild-type bladders, RT-PCR was performed on RNA extracted from E15.5 wild-type bladders using primers specific to *ΔNp63* and *TAp63*, respectively. This analysis confirmed that the predominant N-terminal isoform of *p63* during early bladder development was *ΔNp63* (Fig. 2M). Unlike in the skin, where *p63*α is the predominant C-terminal isoform (Westfall et al., 2003; Yang et al., 1998), RT-PCR detected only *p63*γ and *p63*β in the bladder epithelium (Fig. 2M). Thus, we concluded that *ΔNp63*γ and *ΔNp63*β are the predominant isoforms of *p63* expressed in bladder epithelium during development.

p63 expression is ventrally restricted during early bladder development

To understand better why *p63*$^{-/-}$ embryos develop ventral midline defects, the *p63* expression pattern during early bladder development was studied using immunohistochemistry. Although there was widespread *p63* expression throughout the skin and urothelium in E18.5 embryos (data not shown), sagittal sections of E11.5 embryos showed that *p63* expression was restricted to the ventral epithelia of the urogenital tubercle and UGS, the tail bud, the oral epithelium (Fig. 3A, arrows), and the apical ectodermal ridge (data not shown). Horizontal pelvic sections of E11.5 embryos confirmed that *p63* expression was present in the skin overlying the urogenital tubercle (Fig. 3B) and ventral UGS epithelium (Fig. 3C). In later gestational-stage embryos, *p63* expression was stronger and epithelial stratification was more advanced in the ventral skin compared with that of the dorsal skin (Fig. 3D-F). In summary, *p63* expression in early bladder- and skin-epithelia development is ventrally restricted.

p63-deficient bladder epithelium is abnormal along the dorso-ventral axis

To determine whether the stratification of the endoderm-derived urothelium is affected similarly to that of the ectoderm-derived epithelium in the absence of *p63*, E18.5 *p63*$^{-/-}$ bladders were stained with hematoxylin and Eosin. This analysis revealed that, whereas wild-type bladder epithelium differentiates into stratified transitional urothelium (Fig. 4A,C), the bladder epithelium of *p63*$^{-/-}$ mutants fails to stratify and remains as a single layer (Fig. 4B,D). Differences in epithelial morphology were also noted along the dorso-ventral axis. The dorsal epithelium of *p63*$^{-/-}$ bladder consisted mainly of simple cuboidal cells (Fig. 4D), whereas the ventral epithelial cells were primarily simple squamous cells (Fig. 4B, arrow).

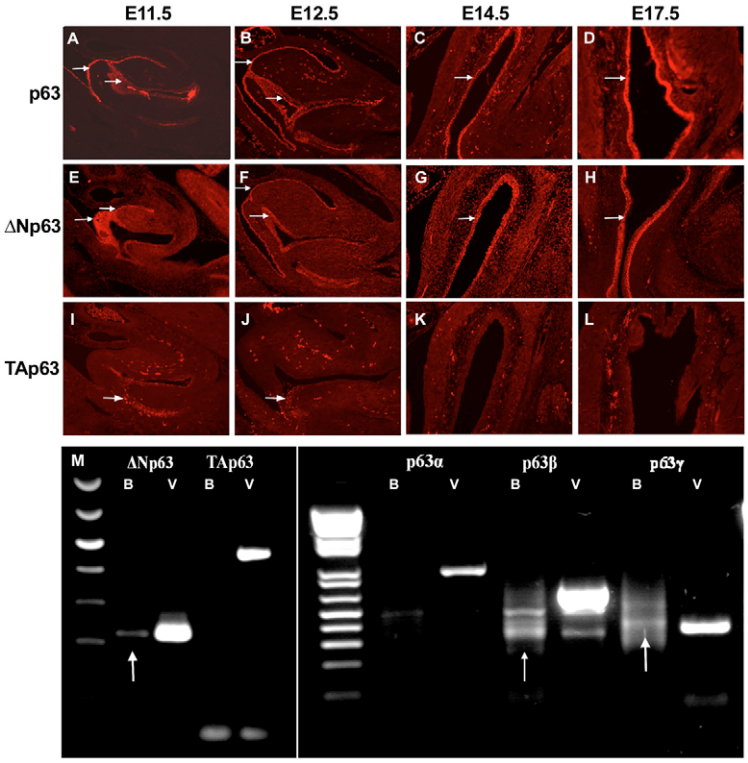

Fig. 2. Ontogeny of *p63* on sagittal sections of wild-type embryos (fluorescent immunohistochemistry, 100×). Sagittal sections of E11.5 embryos transect the epithelium tangentially at the distal UGS, accounting for the wider expression pattern at the distal urogenital sinus (A,E). (**A-D**) *p63* (4A4) expression. (**E-H**) *ΔNp63* isoform expression. (**I-L**) TAp63 isoform expression. Blood cells within the mesenchyme are autofluorescent. (**M**) RT-PCR of wild-type E15.5 bladder, using adenoviruses with *ΔNp63, TAp63*, *p63*α, *p63*β and *p63*γ constructs as controls. Arrows in A-J and M represent the positive immunoreactivities and RT-PCR bands of wild-type bladder samples. B, wild-type bladder cDNA; V, adenoviruses containing *ΔNp63, TAp63*, *p63*α, *p63*β and *p63*γ constructs.

As *p63* has previously been shown to be essential in ectodermal epithelial commitment and/or differentiation (Mills et al., 1999), the role of *p63* in bladder development was examined using well-established markers for epithelial differentiation in E18.5 embryos. K18, a marker of the endoderm or uncommitted epithelium, is not expressed in stratified or differentiated epithelia (Koster et al., 2004). We noted that, in mature wild-type bladder urothelium, K18 expression was absent (Fig. 4E,G). In the *p63*$^{-/-}$ bladder, whereas the dorsal epithelium did not express K18 (Fig. 4H), the ventral epithelium retained K18 expression, indicating that it was uncommitted to stratification (Fig. 4F). To further determine the status of epithelial differentiation in bladder tissue, the expression of uroplakin 3, a marker for terminally differentiated urothelium, was studied (Wu et al., 1999). Uroplakin 3 was strongly expressed in mature wild-type bladder urothelium (Fig. 4I,K). In the *p63*$^{-/-}$ bladder, uroplakin expression was reduced in the dorsal epithelium (Fig. 4L) and undetectable in the ventral epithelium (Fig. 4J). This suggests that, whereas the *p63*$^{-/-}$ ventral bladder epithelium is undifferentiated, the dorsal epithelium is capable of differentiation, even in the absence of *p63* (Fig. 4K,L). As null mutation of *p63* has been reported to be associated with intestinal metaplasia (Signoretti et al., 2005; Yang et al., 1999), intestinal markers were also examined in the *p63*$^{-/-}$ bladder epithelium. This analysis revealed that intestinal transformation does not occur in the *p63*$^{-/-}$ bladder epithelium (Fig. S1 in the supplementary material). In summary, null mutation of *p63* was noticed to affect the development of bladder epithelium differentially along the dorso-ventral axis, ultimately resulting in uncommitted and undifferentiated ventral bladder epithelium.

Apoptosis is increased in p63-deficient bladder epithelium

ΔNp63 is known to act as a naturally occurring dominant negative. It has been shown to counteract the pro-apoptotic actions of *TAp63* and *p53* in vitro (Yang et al., 1998). We have shown that *ΔNp63* is the major isoform of *p63* expressed in the developing ventral

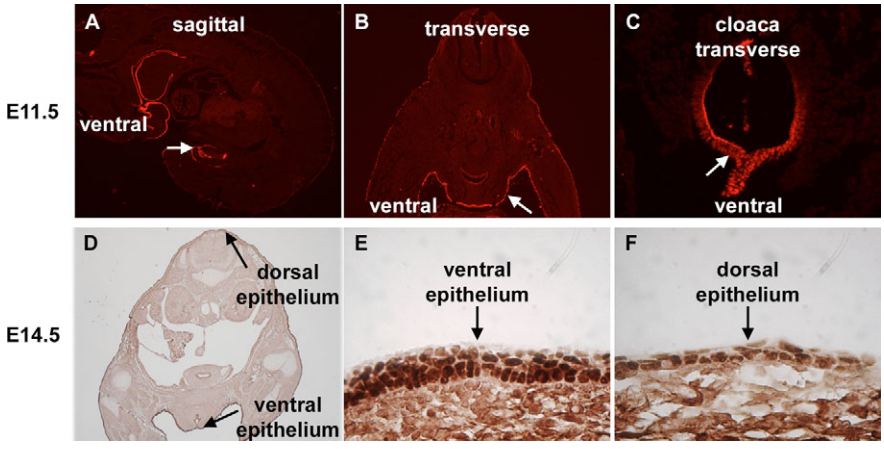

Fig. 3. *p63* (4A4) expressions in E11.5 and E14.5 wild-type embryos. (**A**) *p63* immunofluorescent staining of a sagittal section of an E11.5 embryo (20×). (**B**) *p63* immunofluorescent staining of transverse sections of an E11.5 embryo pelvis (40×). (**C**) *p63* immunofluorescent staining of an E11.5 UGS (200×). Arrows in A-C indicate the ventral aspects of the embryos. (**D**) Colorimetric immunostaining of a transverse section from an E14.5 embryo (20×). High-magnification view of ventral (**E**) and dorsal (**F**) skin of the E14.5 embryo (600×).

bladder, and that both the mesenchyme and epithelium of the ventral UGS develop abnormally in the absence of *ΔNp63* (Fig. 5A,B). As such, we hypothesized that *p63* acts as a pro-survival protein in the developing bladder, thus preventing the apoptosis of ventral UGS epithelium during development. To directly test this hypothesis, we examined the amount of apoptosis in *p63*$^{-/-}$ bladders by TUNEL assay and cleaved caspase-3 expression. The number of TUNEL-positive cells in the ventral UGS epithelium of E11.5 *p63*$^{-/-}$ mutants was significantly higher than that of wild-type controls (Fig. 5C,D) (44% versus 9%, Student's *t*-test, $P<0.05$). This increase in apoptosis was further corroborated by an observed increase in cleaved caspase-3 expression in *p63*$^{-/-}$ ventral UGS epithelium (35% versus 5%, Student's *t*-test, $P<0.05$) (Fig. 5E,F). In comparison, we noted minimal apoptotic activity in the dorsal epithelia of both wild-type and *p63*$^{-/-}$ bladders, as determined by TUNEL assay and cleaved caspase-3 expression (Fig. 5C-F). We also compared the apoptotic

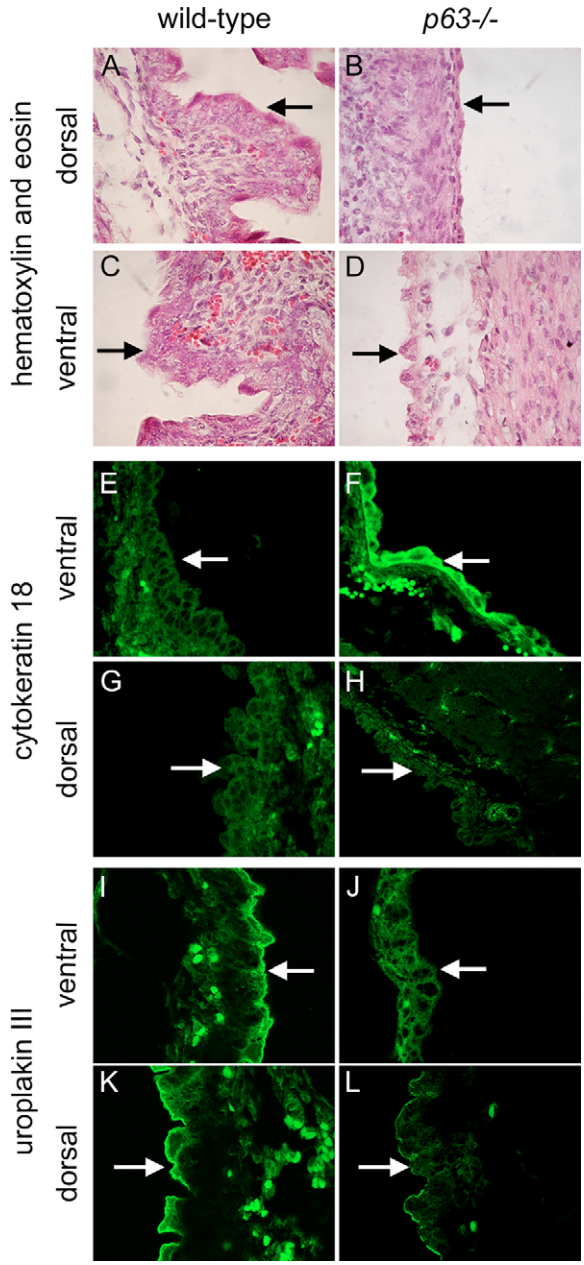

Fig. 4. Ventral and dorsal epithelia of E18.5 wild-type and *p63*$^{-/-}$ bladders. (**A-D**) Hematoxylin and Eosin staining (600×). (**E-H**) Immunofluorescent staining of cytokeratin 18 (K18) (400×, confocal microscopy). (**I-L**) Immunofluorescent staining of uroplakin 3 (630×, confocal microscopy). (A,C,E,G,I,K) Wild type. (B,D,F,H,J,L) p63$^{-/-}$. Arrows represent the epithelial layers.

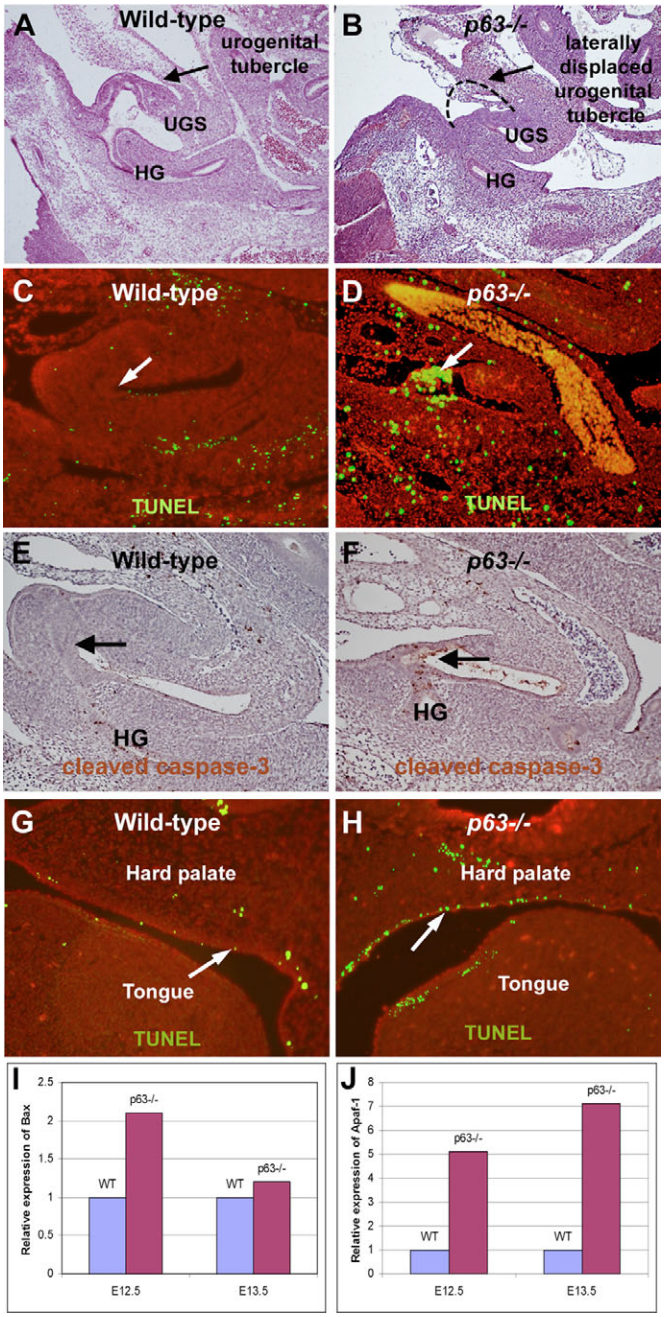

Fig. 5. (**A**,**B**) Hematoxylin and Eosin staining of the sagittal sections of E11.5 wild-type and *p63*$^{-/-}$ embryos (100×). (**C**,**D**) Fluorescent TUNEL staining (arrows) of wild-type and *p63*$^{-/-}$ UGS (200×). The DAPI staining of nuclei is shown in red to increase color contrast. (**E**,**F**) The colorimetric immunostaining (arrows) for cleaved caspase-3 in E11.5 wild-type and *p63*$^{-/-}$ UGS (200×). (**G**,**H**) Fluorescent TUNEL staining (arrows) of sagittal sections through the oral cavity of E11.5 wild-type and *p63*$^{-/-}$ embryos (400×). (**I**,**J**) The qPCR relative expressions of *Bax* and *Apaf1* in E12.5 and E13.5 *p63*$^{-/-}$ bladders. (A,C,E,G) Wild type. (B,D,F,H) p63$^{-/-}$. HG, hindgut.

activities (percentage of cleaved caspase-3-positive cells) of skin overlying the *p63*$^{-/-}$ and wild-type urogenital tubercles (12.8±2.7% and 2.7±1.7%) and found the difference between them was statistically significant (Student's *t*-test, $P<0.01$). This phenomenon of increased apoptosis in the absence of *p63* does not appear to be restricted to the ventral UGS. Oral-cavity epithelium, which also expresses high levels of *p63* during early development (Fig. 3A), was noted to have a statistically significant increase in apoptosis in the *p63*$^{-/-}$ embryo (Fig. 5G,H, 49.0±1.0% versus 9.0±1.0%, Student's *t*-test, $P<0.05$). In summary, our data show that apoptosis is increased in the epithelia of the ventral UGS, as well as in other epithelial structures where *p63* expression is normally high during early development.

Developmental apoptosis, important in normal organogenesis, is understood to involve the mitochondrial apoptotic pathway (Vaux and Korsmeyer, 1999). As such, we analyzed the expression of the mitochondrial apoptotic mediators *Bax* and *Apaf1* in E12.5 and E13.5 wild-type and *p63*$^{-/-}$ bladders by qPCR. In the *p63*-deficient bladders, the relative expressions of *Bax* and *Apaf1* were increased at both E12.5 and E13.5 (Fig. 5I,J). Taken together, our data showed that, in the absence of *ΔNp63*, there was increased mitochondrial apoptotic activity in the developing bladder.

ΔNp63 is anti-apoptotic during bladder development

To confirm the anti-apoptotic role of *ΔNp63* during bladder development, E13.5 *p63*$^{-/-}$ bladder primary cell cultures were infected with bicistronic adenoviruses expressing *TAp63*γ, *ΔNp63*β, *ΔNp63*γ and green fluorescent protein (GFP), or GFP alone, for 24 hours. The cells were then harvested and the expressions of *Bax* and *Apaf1* were quantified with immunoblots. Their gel tensiometry readings were compared. Compared with the GFP-transfected control, transfection with *ΔNp63*β or *ΔNp63*γ adenoviruses significantly reduced the expressions of both *Bax* ($P<0.05$) and *Apaf1* ($P<0.01$), whereas transfection with *TAp63*γ adenovirus led to an increase in the expressions of both *Bax* and *Apaf1* ($P<0.05$) (Fig. 6A,B). To confirm the anti-apoptotic role of *ΔNp63,* organ cultures of E13.5 *p63*$^{-/-}$ bladders were infected with bicistronic adenoviruses expressing *ΔNp63*γ and GFP or GFP alone for 24 hours. *Bax* expression was examined by qPCR. We observed a more than 50% reduction of *Bax* relative expression in the *ΔNp63*γ-infected *p63*$^{-/-}$ bladder. These data suggest that *ΔNp63*, the predominant isoform of *p63* in the bladder, plays an anti-apoptotic role during bladder development.

Apoptosis of bladder cells of E12.5 *p63*$^{-/-}$ animals is associated with upregulation of p53 and p73 expression

We then examined whether the expressions of *p53* and *p73* were affected in *p63*$^{-/-}$ bladders. Immunohistochemical staining showed an upregulation of p53 expression in the ventral aspect of E11.5 *p63*$^{-/-}$ UGS (Fig. 6C,D). Additionally, there also appeared to be an upregulation of p73 expression in *p63*$^{-/-}$ UGS (Fig. 6E,F). We proceeded to quantify the *p53* and *p73* mRNA expressions in E12.5 and E13.5 wild-type and *p63*$^{-/-}$ bladders using real-time PCR analysis. There was a compensatory upregulation of the relative expressions of both *p53* and *p73* in E12.5 *p63*$^{-/-}$ bladders, compared with those of the wild-type controls (Student's *t*-test, $P<0.05$). Interestingly, in E13.5 *p63*$^{-/-}$ bladders, the expressions of both *p53* and *p73* were downregulated (Fig. 6G,H, Student's *t*-tests, $P<0.01$). The transient upregulation of *p53* and *p73* expression in *p63*$^{-/-}$ bladders co-incides temporally with increased apoptosis (Fig. 5C-F).

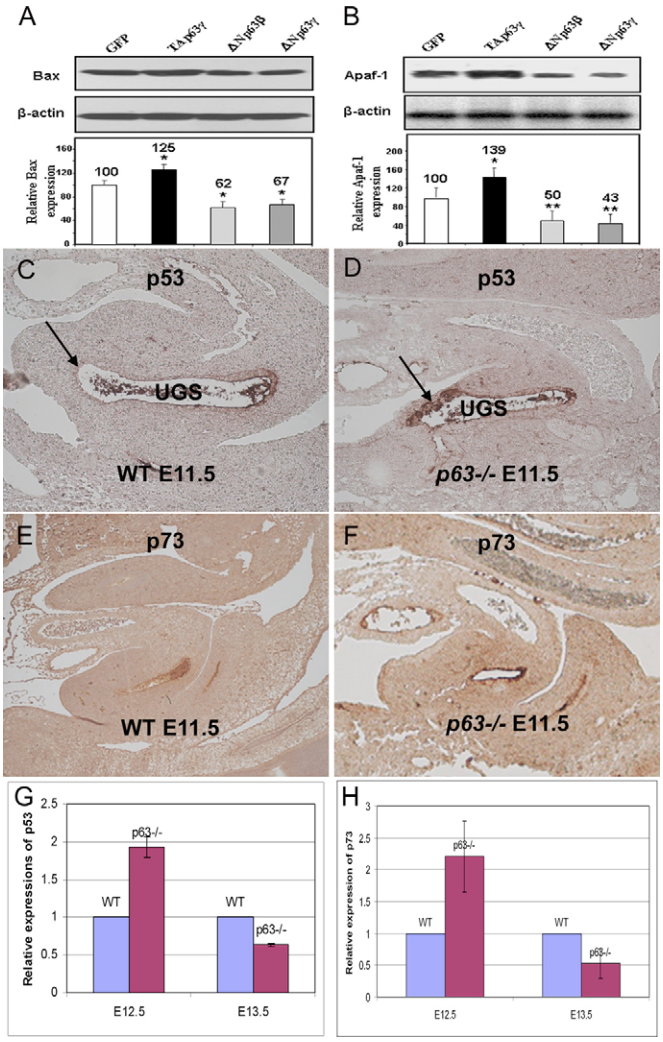

Fig. 6. The E13.5 *p63*$^{-/-}$-bladder primary cell cultures were transfected with adenoviruses expressing GFP, *TAp63*γ, *ΔNp63*β *and ΔNp63*γ. The tensiometry of *Bax*, *Apaf1* and β-actin bands was recorded. The ratios of *Bax*/β-actin and *Apaf1*/β-actin of the specimens were compared with that of the GFP-infected controls (assigned to be 100). (**A**,**B**) Relative *Bax*/β-actin (A) and *Apaf1*/β-actin (B) ratios (Student's *t*-test, **P*<0.05, ***P*<0.01). (**C**,**D**) Immunohistochemical staining of p53 in E11.5 wild-type (C) and *p63*$^{-/-}$ (D) UGS (200×). Arrows represent the ventral UGS. (**E**,**F**) Immunohistochemical staining of p73 in E11.5 wild-type (E) and *p63*$^{-/-}$ (F) UGS (100×). (**G**,**H**) The qPCR relative expressions of *p53* (G) and *p73* (H) in E12.5 and E13.5 wild-type (WT) and *p63*$^{-/-}$ bladders.

Failure of ventral UGS mesenchymal induction and proliferation in the absence of epithelial ΔNp63

Appropriate epithelial-mesenchymal interaction is essential for normal bladder development; in the absence of bladder epithelium, bladder smooth muscle does not develop normally (Baskin et al., 1996a). To examine how the *p63*-null epithelium affects the adjacent mesenchyme, we studied the expression of *Msx1*, a homeobox gene that is induced in the mesenchyme by an epithelium-derived signal. In the wild-type control embryos, *Msx1* was expressed in the ventral subepithelial mesenchyme of E13.5 bladders, suggesting expression of *Msx1* in the mesenchyme by the

ventral UGS epithelium (Fig. 7A, arrow). By contrast, the expression of *Msx1* is greatly reduced or absent in the sub-epithelial mesenchyme of E13.5 $p63^{-/-}$ bladders (Fig. 7B). To assess whether the *p63*-positive epithelium release mediator(s) that are crucial for homeostasis of the mesenchyme, we studied, by mRNA in situ hybridization, the expression of *Fgf8*, another marker normally induced in the apical ectodermal ridge by *p63* (Mills et al., 1999; Yang et al., 1999). *Fgf8* is normally expressed in the mesenchyme of E11.5 wild-type UGS (Maruoka et al., 1998) (Fig. 7C). We found that the expression of *Fgf8* was downregulated in the $p63^{-/-}$ ventral UGS (Fig. 7D). These results suggest that *p63* deficiency in UGS epithelium is associated with a failure of induction in the adjacent UGS mesenchyme, especially ventrally. Epithelial-mesenchymal transition (EMT) is a cellular mechanism during which certain cells switch from an epithelial to a mesenchymal status. During development, EMT is involved in neural-crest migration, heart morphogenesis and formation of palate mesenchymal cells from the oral epithelium on E13.5 in mice (Larue and Bellacosa, 2005). We questioned whether bladder epithelial cells undergo EMT, as this might explain why the failure of epithelial development is associated with failure of mesenchymal development. We studied the expression of Snai1 (m-snail), which induces the epithelial-mesenchymal transition (Barrallo-Gimeno and Nieto, 2005; Cano et al., 2000). We could not detect any Snai1 expression in either the wild-type or $p63^{-/-}$ E14.5 bladders by in situ hybridization (Fig. 7E,F). These results failed to demonstrate the role of EMT at this stage of bladder development.

To explain the paucity of smooth muscle in the ventral bladder wall, mesenchymal cell proliferation was studied with the incorporation of BrdU. *Msx1* is commonly expressed in regions of rapid proliferation (Bendall and Abate-Shen, 2000) and Fgf8 regulates survival and proliferation in the anterior heart field (Park et al., 2006). In the absence of epithelial *ΔNp63* expression, mesenchymal expression of both *Msx1* and *Fgf8* were decreased. This was accompanied by a reduction in cell proliferation in both the epithelium and mesenchyme, especially ventrally (Fig. 7G,H). The difference in cell proliferation between $p63^{-/-}$ and the wild-type control was statistically significant (ANOVA, dorsal epithelium and mesenchyme: $P<0.01$; ventral epithelium and ventral mesenchyme: $P<0.0001$) (Fig. 7I). Taken together, in the absence of *ΔNp63*, the ventral bladder epithelium fails to induce expression of *Msx1* and *Fgf8* in the adjacent mesenchyme. This is associated with a decreased mesenchymal cell proliferation.

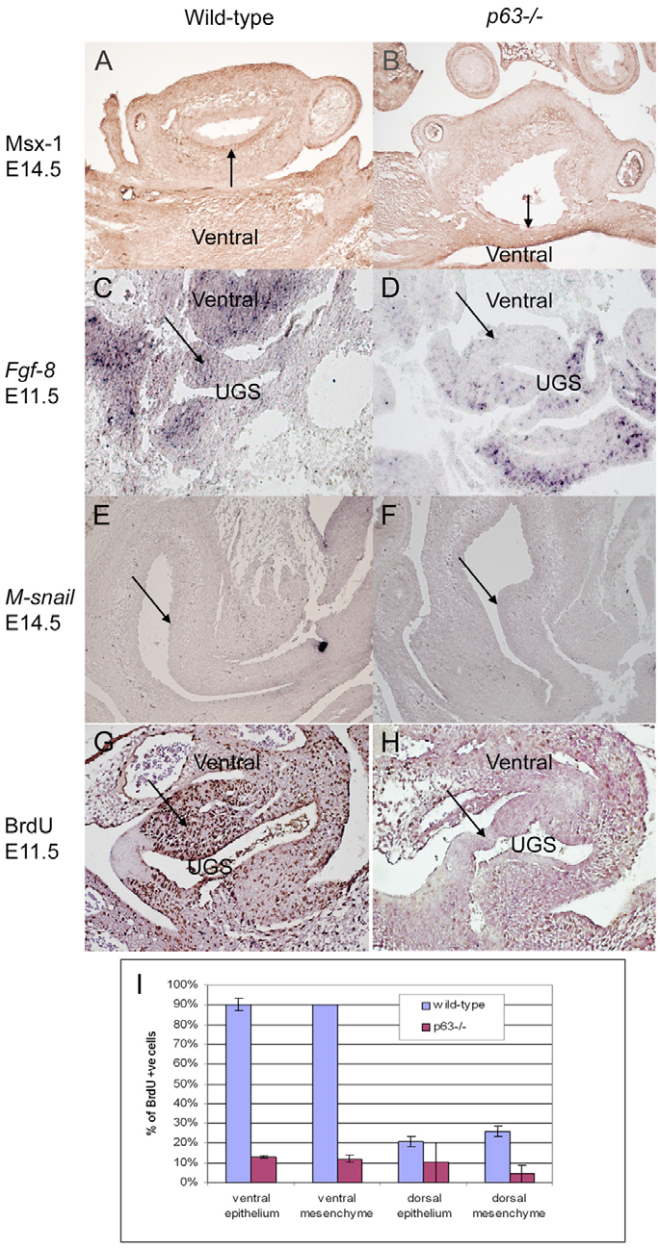

Fig. 7. Epithelial-mesenchymal interactions. (**A**,**B**) *Msx1* expressions (immunohistochemistry) in transverse sections of E14.5 wild-type (A) and $p63^{-/-}$ (B) bladders (100×). (**C**,**D**) *Fgf8* in situ hybridization in the sagittal sections of E11.5 wild-type (C) and $p63^{-/-}$ (D) UGS (100×). (**E**,**F**) *m-snail* in situ hybridization of E14.5 wild-type (E) and $p63^{-/-}$ (F) bladders (sagittal sections) (100×). (**G**,**H**) BrdU incorporation in the sagittal sections of wild-type (G) and $p63^{-/-}$ (H) E11.5 UGS (100×). Arrows in A-H represent the ventral UGS. (**I**) Histogram of cell proliferation in both epithelium and mesenchyme of E11.5 wild-type and $p63^{-/-}$ UGS.

Smooth-muscle differentiation is disturbed in p63-deficient bladders

Msx1 is known to repress terminal differentiation (Bendall and Abate-Shen, 2000). To determine the effect of *Msx1* downregulation on smooth-muscle differentiation, the expression of smooth-muscle heavy-chain myosin, which is present only in mature smooth-muscle cells (Owens, 1995), was studied immunohistochemically. In E14.5 wild-type bladders, smooth-muscle heavy-chain myosin expression was absent or very weak, whereas, in the $p63^{-/-}$ bladder, its expression was strong in the thin ventral bladder wall (Fig. 8A,B). This suggests that the absence of Msx1 in the ventral mesenchyme allowed premature smooth-muscle differentiation in the adjacent mesenchyme. Despite premature smooth-muscle differentiation, the E18.5 $p63^{-/-}$ bladder contained little or no smooth muscle ventrally, but did retain a thin layer of smooth muscle dorsally (smooth-muscle α-actin). Moreover, the lamina propria was either greatly reduced or absent in the $p63^{-/-}$ bladder (Fig. 8C,D). In addition, unlike the wild-type bladder detrusor muscle, which displayed well-organized smooth-muscle stratification, the dorsal smooth muscle in the $p63^{-/-}$ bladder was disorganized and non-stratified (Fig. 8E,F).

DISCUSSION

Our experimental findings support a number of conclusions. First, *ΔNp63* is preferentially expressed in the ventral UGS during early bladder development. Second, in the absence of *ΔNp63*, ventral

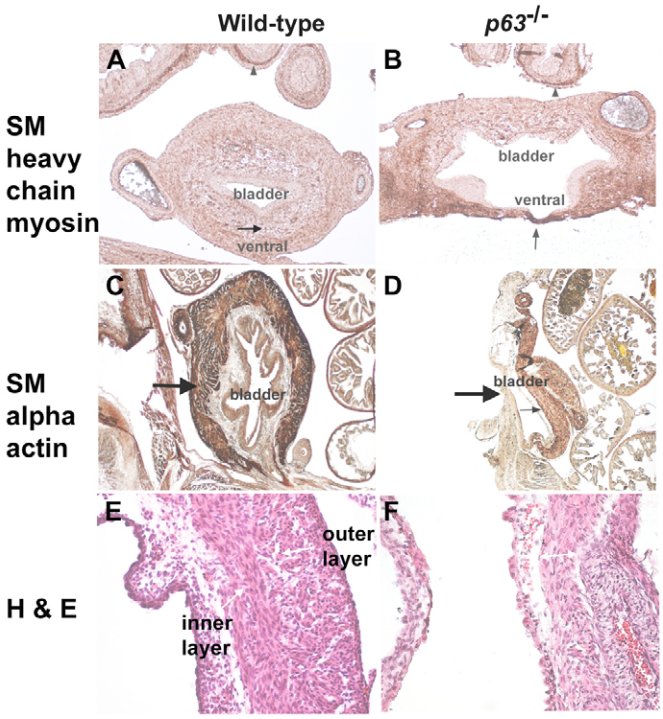

Fig. 8. Bladder smooth-muscle development. (**A**,**B**) Smooth-muscle heavy-chain myosin expressions in the transverse sections of the E14.5 wild-type (A) and *p63*$^{-/-}$ (B) bladders. The intestinal muscular wall serves as an internal control (arrow, 100×). (**C**,**D**) Smooth-muscle α-actin actin expressions in the sagittal section of E18.5 wild-type (C) and *p63*$^{-/-}$ (D) bladders (40×). Large arrow in C,D represent the ventral bladder walls. Small arrow in D represents the dorsal bladder wall. (**E**,**F**) Hematoxylin and Eosin staining of E18.5 wild-type (E) and *p63*$^{-/-}$ (F) bladders (sagittal sections, 200×).

bladder epithelial development is abnormal because of an increase in ventral bladder epithelial apoptosis. Third, *ΔNp63* prevents this ventral bladder epithelial apoptosis by, at least partially, downregulating the mitochondrial apoptotic pathway. Finally, in the absence of *ΔNp63*, there is decreased cell proliferation in the UGS mesenchyme. The increased epithelial apoptosis and decreased cell proliferation in both epithelium and mesenchyme ultimately results in bladder exstrophy in *p63*$^{-/-}$ embryos.

Early ventral p63 expression and ventral midline defects in p63$^{-/-}$ mutants

In the current study, we demonstrated that, during early development, *p63* is preferentially expressed in the epithelia of ventral structures, including the genital tubercle (Fig. 3B), oral cavity (Yang et al., 1999) and ventral UGS (Fig. 3C) (Kurita et al., 2004a). In the absence of *p63*, development of these ventral structures is defective, and is manifested as a truncated maxilla, cleft palate, ventral pelvic-wall defect (Ince et al., 2002), bifid genitalia and bladder exstrophy. We have established that *ΔNp63* is the predominant *p63* isoform throughout bladder development. In a zebrafish model, *ΔNp63* has been noted to be required for ventral specification, with loss of *ΔNp63* resulting in a reduction of ventral (non-neural) ectoderm, whereas overexpression of *ΔNp63* expands the ventral ectoderm (Bakkers et al., 2002). *ΔNp63* is also a direct target of Bmp4 (Bakkers et al., 2002), a morphogen vital for correct ventral patterning (Lemaire and Yasuo, 1998). In light of these findings, our results suggest that *ΔNp63* may be a ventralizing protein in mammalian development and absence of *ΔNp63* may account for the ventral midline defects observed in *p63*$^{-/-}$ embryos.

Msx1 is a mesenchymal marker, the expression of which is induced by adjacent epithelial tissue (Jowett et al., 1993). In *p63*$^{-/-}$ embryos, in which limb buds are absent or vestigial, *Msx1* expression in the progress zone beneath the apical ectodermal ridge of the limb bud is greatly reduced or absent (Mills et al., 1999; Yang et al., 1999). Our findings further suggest that epithelial-mesenchymal interaction also plays an important role in ventral bladder development. *Msx1* expression in the ventral mesenchyme is deficient in the *p63*$^{-/-}$ bladder, suggesting that *p63*-deficient epithelium fails to induce appropriately the adjacent mesenchyme (Fig. 6A,B). This decreased *Msx1* expression is associated with a reduction in UGS mesenchymal proliferation and premature terminal differentiation of the smooth muscle. Interestingly, Msx1 is also a ventralizing signal responsible for mesoderm patterning under the regulation of Bmp4 in *Xenopus* (Takeda et al., 2000). In summary, a failure of mesenchymal induction may be responsible for the changes observed in *p63*$^{-/-}$ ventral UGS mesenchyme.

Notably, the specific epithelial signal to the UGS mesenchyme remains undefined. A possible candidate protein for this role is the secreted diffusible morphogen *sonic hedgehog* (*Shh*). *Shh* is known to participate in numerous developmental processes involving the epithelial-mesenchymal interaction (Ingham and McMahon, 2001). It also promotes proliferation and inhibits differentiation in renal mesenchymal cell development (Yu et al., 2002). Furthermore, *Shh* is expressed in the UGS epithelium during early bladder organogenesis (Bitgood and McMahon, 1995; Mo et al., 2001). We found that the expression of *Shh* was reduced in the ventral *p63*$^{-/-}$ UGS, where the epithelium is squamous and uncommitted (Fig. S2 in the supplementary material). The reduction of *Shh* signaling may contribute to the reduction in cell proliferation and premature terminal differentiation in ventral bladder mesenchymal development. This remains to be determined.

Temporospatial restriction of p63 expression determines epithelial commitment to stratification and differentiation

The exact role of *p63* during epithelial development is controversial (McKeon, 2004). *p63* either commits the epithelium to stratification (Mills et al., 1999) or maintains epithelial proliferation (Yang et al., 1999). Koster et al. suggested that *TAp63* initiates epithelial commitment and that *ΔNp63* is responsible for epithelial differentiation (Koster et al., 2004). Our study shows that, during the developmental period examined (E11.5-E17.5), *ΔNp63* expression began in the ventral UGS and progressively extended to the remaining bladder urothelium. In the *p63*$^{-/-}$ bladder, a clear phenotypic difference is noted in the epithelium along the dorso-ventral axis of the bladder. The ventral epithelium is squamous, with almost no adjacent smooth muscle, whereas the dorsal epithelium is cuboidal with a thin layer of disorganized muscle. The distal ventral UGS epithelium, which is destined to become the urethra, transforms into an intestine-like epithelium (Signoretti et al., 2005). The ventral epithelium remains both uncommitted (positive for K18) and undifferentiated (negative for uroplakin 3). The dorsal epithelium, however, is both committed (negative for K18) and differentiated (positive for uroplakin 3). These results suggest that the timing of *p63* expression in normal bladder development determines the extent of developmental delay. Thus, the absence of *p63* during early ventral bladder development affects both epithelial

commitment and differentiation, whereas, in dorsal epithelium, where *p63* is normally expressed later, *p63* deletion does not appear to affect either commitment or differentiation.

ΔNp63 is prosurvival in ventral bladder development

This study provides in vivo evidence that *ΔNp63* is anti-apoptotic during bladder development. In vitro study showed that *ΔNp63* can compete for the apoptotic target-gene site or form a transactivation-incompetent heterocomplex with *p53* or *TAp73*, thus inhibiting apoptosis (Yang et al., 2002). In our study, apoptotic activity was increased in the ventral UGS epithelium in the absence of *ΔNp63* (Fig. 5D,F). Expression of the mitochondrial apoptotic mediators *Bax* and *Apaf1* was also elevated in $p63^{-/-}$ bladders; elevated *Bax* and *Apaf1* expressions in $p63^{-/-}$ bladders were rescued by the overexpression of either *ΔNp63*β or *ΔNp63*γ. This rescue is corroborated by a previous study, in which ectopic *ΔNp63*α expression in the epidermis reduces epidermal susceptibility to ultraviolet light-induced apoptosis (Liefer et al., 2000). In developing sympathetic neurons, where *ΔNp73* is the predominant isoform, a *p73* knockout leads to increased apoptosis in a fashion similar to the *p63* knockout in the ventral bladder. Overexpression of *ΔNp73* rescues the sympathetic neurons from apoptosis induced by withdrawal of the nerve growth factor (Pozniak et al., 2000). Our results not only confirm the functional consistency of ΔN isoform proteins of the *p53* family, but also demonstrate the anti-apoptotic role of the *ΔNp63* isoform during normal mammalian development.

ΔNp63 is also detected in oral carcinoma and the intensity of its expression increases with the severity of dysplasia (Nylander et al., 2002), suggesting an oncogenic role or stem-cell pluripotency factor for the *ΔNp63* isoform. The possible mechanism may involve a *p53* target gene, *p21*, as *ΔNp63*α binds the *p21* promoter, represses its transcription and permits cell-cycle progression (Westfall et al., 2003). The scenario is similar to *ΔNp53*, which is tumorigenic (Mowat et al., 1985). Overexpression of a C-terminal dominant-negative fragment of *p53* (*ΔNp53*) (Shaulian et al., 1992) in human urothelial cells has been reported to increase the cell-proliferation rate (Shaw et al., 2005). In this study, $p63^{-/-}$ mutation has been shown to be associated with a significant reduction of cell proliferation in the ventral bladder epithelium. This suggests that *ΔNp63* also promotes epithelial proliferation in mammalian bladder development. Autocrine regulation of urothelial cell proliferation via the EGFR signaling loop observed in urothelial regenerative response could also play a role in urothelial development (Varley et al., 2005).

Our data showed that deletion of *p63* is associated with compensatory upregulation of *p53* expression in the bladders of younger embryos (E11.5-E12.5). This *p53* upregulation may further contribute to the apoptosis observed in the ventral $p63^{-/-}$ bladder, in addition to the protein-protein and protein-target gene interactions. Although the expression of *p73* in $p63^{-/-}$ bladders is also upregulated, its role in inducing apoptosis is uncertain, as the predominant isoform of p73 expressed in bladder has not been studied.

In conclusion, we have established a $p63^{-/-}$ murine model for BE. We have found that *ΔNp63* is expressed initially in the ventral bladder urothelium and possesses a ventralizing property. Although the complete bladder urothelium fails to stratify in the absence of *p63*, ventral urothelial development is more delayed than that in the dorsal epithelium, being both uncommitted and undifferentiated. We found that *ΔNp63* is the predominant isoform in the bladder. Without *ΔNp63*, urothelial apoptosis is increased and cell proliferation is reduced. We also noted a concurrent upregulation of *p53* expression. Overexpression of *ΔNp63*γ and *ΔNp63*β rescue the expression levels of the mitochondrial apoptotic mediators *Bax* and *Apaf1* in $p63^{-/-}$ bladders. We conclude that *ΔNp63* plays a crucial anti-apoptotic role during ventral bladder development.

This work was supported by grants from the Canadian Institute of Health Research (#57889) and March of Dimes Birth Defects Foundation, USA (#FY02-154).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/23/4783/DC1

References

Bakkers, J., Hild, M., Kramer, C., Furutani-Seiki, M. and 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.

Barrallo-Gimeno, A. and Nieto, M. A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132**, 3151-3161.

Baskin, L. S., Hayward, S. W., Sutherland, R. A., DiSandro, M. J., Thomson, A. A., Goodman, J. and Cunha, G. R. (1996a). Mesenchymal-epithelial interactions in the bladder. *World J. Urol.* **14**, 301-309.

Baskin, L. S., Hayward, S. W., Young, P. and Cunha, G. R. (1996b). Role of mesenchymal-epithelial interactions in normal bladder development. *J. Urol.* **156**, 1820-1827.

Bendall, A. J. and Abate-Shen, C. (2000). Roles for Msx and Dlx homeoproteins in vertebrate development. *Gene* **247**, 17-31.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

Boyadjiev, S. A., Dodson, J. L., Radford, C. L., Ashrafi, G. H., Beaty, T. H., Mathews, R. I., Broman, K. W. and Gearhart, J. P. (2004). Clinical and molecular characterization of the bladder exstrophy-epispadias complex: analysis of 232 families. *BJU Int.* **94**, 1337-1343.

Cano, A., Perez-Moreno, M., Rodrigo, I., Locascio, A., Blanco, M., de Barrio, M., Portillo, F. and Nieto, A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76-83.

Hui, C. C. and Joyner, A. L. (1993). A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat. Genet.* **3**, 241-246.

Ince, T. A., Cviko, A. P., Quade, B. J., Yang, A., McKeon, F. D., Mutter, G. L. and Crum, C. P. (2002). p63 Coordinates anogenital modeling and epithelial cell differentiation in the developing female urogenital tract. *Am. J. Pathol.* **161**, 1111-1117.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Jacobs, W. B., Govoni, G., Ho, D., Atwal, J. K., Barnabe-Heider, F., Keyes, W. M., Mills, A. A., Miller, F. D. and Kaplan, D. R. (2005). P63 is an essential proapoptotic protein during neural development. *Neuron* **48**, 743-756.

Jowett, A. K., Vainio, S., Ferguson, M. W., Sharpe, P. T. and Thesleff, I. (1993). Epithelial-mesenchymal interactions are required for msx 1 and msx 2 gene expression in the developing murine molar tooth. *Development* **117**, 461-470.

Koster, M. I., Kim, S., Mills, A. A., DeMayo, F. J. and Roop, D. R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev.* **18**, 126-131.

Kurita, T. and Cunha, G. R. (2001). Roles of p63 in differentiation of Mullerian duct epithelial cells. *Ann. N. Y. Acad. Sci.* **948**, 9-12.

Kurita, T., Medina, R. T., Mills, A. A. and Cunha, G. R. (2004a). Role of p63 and basal cells in the prostate. *Development* **131**, 4955-4964.

Kurita, T., Mills, A. A. and Cunha, G. R. (2004b). Roles of p63 in the diethylstilbestrol-induced cervicovaginal adenosis. *Development* **131**, 1639-1649.

Larue, L. and Bellacosa, A. (2005). Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3′ kinase/AKT pathways. *Oncogene* **24**, 7443-7454.

Lemaire, P. and Yasuo, H. (1998). Developmental signalling: a careful balancing act. *Curr. Biol.* **8**, R228-R231.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331.

Liefer, K. M., Koster, M. I., Wang, X. J., Yang, A., McKeon, F. and Roop, D. R. (2000). Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res.* **60**, 4016-4020.

Martinez-Frias, M. L., Bermejo, E., Rodriguez-Pinilla, E. and Frias, J. L. (2001).

Exstrophy of the cloaca and exstrophy of the bladder: two different expressions of a primary developmental field defect. *Am. J. Med. Genet.* **99**, 261-269.
Maruoka, Y., Ohbayashi, N., Hoshikawa, M., Itoh, N., Hogan, B. L. and Furuta, Y. (1998). Comparison of the expression of three highly related genes, Fgf8, Fgf17 and Fgf18, in the mouse embryo. *Mech. Dev.* **74**, 175-177.
McKeon, F. (2004). p63 and the epithelial stem cell: more than status quo? *Genes Dev.* **18**, 465-469.
Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R. and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708-713.
Mo, R., Kim, J. H., Zhang, J., Chiang, C., Hui, C. C. and Kim, P. C. (2001). Anorectal malformations caused by defects in sonic hedgehog signaling. *Am. J. Pathol.* **159**, 765-774.
Moll, U. M. and Slade, N. (2004). p63 and p73: roles in development and tumor formation. *Mol. Cancer Res.* **2**, 371-386.
Mowat, M., Cheng, A., Kimura, N., Bernstein, A. and Benchimol, S. (1985). Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. *Nature* **314**, 633-636.
Muecke, E. C. (1964). The role of the cloacal membrane in exstrophy: the first successful experimental study. *J. Urol.* **92**, 659-667.
Murray-Zmijewski, F., Lane, D. P. and Bourdon, J. C. (2006). p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ.* **13**, 962-972.
Nylander, K., Vojtesek, B., Nenutil, R., Lindgren, B., Roos, G., Zhanxiang, W., Sjostrom, B., Dahlqvist, A. and Coates, P. J. (2002). Differential expression of p63 isoforms in normal tissues and neoplastic cells. *J. Pathol.* **198**, 417-427.
Owens, G. K. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75**, 487-517.
Park, E. J., Ogden, L. A., Talbot, A., Evans, S., Cai, C. L., Black, B. L., Frank, D. U. and Moon, A. M. (2006). Required, tissue-specific roles for Fgf8 in outflow tract formation and remodeling. *Development* **133**, 2419-2433.
Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R. and Miller, F. D. (2000). An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. *Science* **289**, 304-306.
Qiu, W., Kohen-Avramoglu, R., Rashid-Kolvear, F., Au, C. S., Chong, T. M., Lewis, G. F., Trinh, D. K., Austin, R. C., Urade, R. and Adeli, K. (2004). Overexpression of the endoplasmic reticulum 60 protein ER-60 downregulates apoB100 secretion by inducing its intracellular degradation via a nonproteasomal pathway: evidence for an ER-60-mediated and pCMB-sensitive intracellular degradative pathway. *Biochemistry* **43**, 4819-4831.
Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A. and Tabin, C. (1995). Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* **121**, 3163-3174.
Shapiro, E., Lepor, H. and Jeffs, R. D. (1984). The inheritance of the exstrophy-epispadias complex. *J. Urol.* **132**, 308-310.
Shaulian, E., Zauberman, A., Ginsberg, D. and Oren, M. (1992). Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. *Mol. Cell. Biol.* **12**, 5581-5592.
Shaw, N. J., Georgopoulos, N. T., Southgate, J. and Trejdosiewicz, L. K. (2005). Effects of loss of p53 and p16 function on life span and survival of human urothelial cells. *Int. J. Cancer* **116**, 634-639.
Signoretti, S., Pires, M. M., Lindauer, M., Horner, J. W., Grisanzio, C., Dhar, S., Majumder, P., McKeon, F., Kantoff, P. W., Sellers, W. R. et al. (2005). p63 regulates commitment to the prostate cell lineage. *Proc. Natl. Acad. Sci. USA* **102**, 11355-11360.
Takeda, M., Saito, Y., Sekine, R., Onitsuka, I., Maeda, R. and Maeno, M. (2000). Xenopus msx-1 regulates dorso-ventral axis formation by suppressing the expression of organizer genes. *Comp. Biochem. Physiol.* **126B**, 157-168.
Varley, C., Hill, G., Pellegrin, S., Shaw, N. J., Selby, P. J., Trejdosiewicz, L. K. and Southgate, J. (2005). Autocrine regulation of human urothelial cell proliferation and migration during regenerative responses in vitro. *Exp. Cell Res.* **306**, 216-229.
Vaux, D. L. and Korsmeyer, S. J. (1999). Cell death in development. *Cell* **96**, 245-254.
Westfall, M. D., Mays, D. J., Sniezek, J. C. and Pietenpol, J. A. (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.
Wu, H. Y., Baskin, L. S., Liu, W., Li, Y. W., Hayward, S. and Cunha, G. R. (1999). Understanding bladder regeneration: smooth muscle ontogeny. *J. Urol.* **162**, 1101-1105.
Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D. and 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.
Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C. et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**, 714-718.
Yang, A., Kaghad, M., Caput, D. and McKeon, F. (2002). On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet.* **18**, 90-95.
Yu, J., Carroll, T. J. and McMahon, A. P. (2002). Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* **129**, 5301-5312.