Inhibitor of apoptosis-stimulating protein of p53 (iASPP) prevents senescence and is required for epithelial stratification

Mario Notari[a,1], Ying Hu[a,1], Sofia Koch[a], Min Lu[a], Indrika Ratnayaka[a], Shan Zhong[a], Caroline Baer[a], Anna Pagotto[a], Robert Goldin[b], Victoria Salter[a], Eleonora Candi[c], Gerry Melino[c,d], and Xin Lu[a,2]

[a]Nuffield Department of Clinical Medicine, Ludwig Institute for Cancer Research, University of Oxford, Oxford OX3 7DQ, United Kingdom; [b]Department of Pathology, Imperial College London, Faculty of Medicine at St. Mary's, Norfolk Place, London W2 1PG, United Kingdom; [c]Biochemistry Laboratory IDI-IRCCS, University of Rome 'Tor Vergata', Rome 00133, Italy; and [d]MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, United Kingdom

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Inhibitor of apoptosis-stimulating protein of p53 (iASPP) is the most ancient member of the ASPP family of proteins and an evolutionarily conserved inhibitor of p53. iASPP is also a binding partner and negative regulator of p65RelA. Because p65RelA and the p53 family members often have opposite effects in controlling cell fate, it is important to understand the cellular context in which iASPP can regulate their activities. To address this question and to study the biological importance of iASPP in vivo, we generated a transgenic mouse in which iASPP expression is controlled by the Cre/loxP recombination system. We observed that iASPP is able to prevent premature cellular senescence in mouse embryonic fibroblasts. iASPP loss resulted in increased differentiation of primary keratinocytes both in vitro and in vivo. In stratified epithelia, nuclear iASPP often colocalized with p63 in the nuclei of basal keratinocytes. Consistent with this, iASPP bound p63 and inhibited the transcriptional activity of both TAp63α and ΔNp63α in vitro and influenced the expression level of p63-regulated genes such as *lor-icrin* and *involucrin* in vivo. In contrast, under the same conditions, p65RelA was frequently expressed as a cytoplasmic protein in the suprabasal layers of stratified epithelia and rarely colocalized with nuclear iASPP. Thus, iASPP is likely to control epithelial stratification by regulating p63's transcriptional activity, rather than p65RelA's. This study identifies iASPP as an inhibitor of senescence and a key player in controlling epithelial stratification.

One of the most conserved biological functions of the p53 family of proteins is their ability to induce apoptosis in response to stress (1). In *Caenorhabditis elegans*, Cep1 is the only p53 family member, and its apoptotic function is inhibited by Ce-inhibitor of apoptosis-stimulating protein of p53 (iASPP) the only member of the ASPP family (2, 3). In vertebrates, both the p53 and ASPP families consist of three members: p53, p63, and p73 for the p53 family and ASPP1, ASPP2, and iASPP for the ASPP family (4). The p53 family members share high sequence similarity in their DNA binding domains and are able to transactivate and transrepress a large number of genes that, in turn, control a number of biological processes including apoptosis and senescence. In theory, the three p53 family members can all bind the same putative p53 binding sites and regulate the same target genes (5). However, transcriptional profiling has demonstrated that, although the transcriptional targets of p53, p63, and p73 overlap, they are not identical (6). This diversity in target selectivity is also reflected in the p53 family's biological functions in vivo. Functionally, p53 is a master sensor of stress and a potent inducer of apoptosis and cellular senescence; hence, it is the most important tumor suppressor and one of the most frequently mutated genes in human cancers (1). p63, however, is a master transcription factor in epithelial stratification (7, 8), whereas p73 is a key regulator of neural development (9).

The ASPP family of proteins was one of the first to be identified as able to alter the promoter selectivity of p53 and its siblings, and specifically regulates p53-induced apoptosis (4). By interacting with their DNA binding domains, ASPP1 and ASPP2 selectively enhance the ability of the p53 family of proteins to transactivate target genes and increase the expression of PIG3, Bax, and PUMA, but not $p21^{waf1/CIP1}$ or mdm2. Thus, ASPP1 and ASPP2 are common activators of p53, p63, and p73 (10). Transgenic mouse studies have shown that ASPP2 is a haploinsufficient tumor suppressor and activator of p53 in vivo (11, 12). However, compared with ASPP1 and ASPP2, much less is known about iASPP's ability to regulate the p53 family. Existing data show that iASPP can bind and selectively inhibit the transcriptional activity of p53 on the promoters of PIG3 and Bax, but not $p21^{waf1/CIP1}$ in tumor cells (3). In vitro, iASPP binds the p53 family members with different affinities: its affinity for p53 is the highest, whereas the affinity of purified iASPP's binding to p63 is three times greater than that for p73 (13). It, therefore, begs the question whether iASPP can bind and regulate p63 in vivo.

In addition to binding p53, the C terminus of iASPP has been shown to interact with p65RelA, and can also inhibit its transcriptional activity (14). Thus, it is unclear whether iASPP can act as an inhibitor of the p63 and p65RelA pathways and, if it can, when and how iASPP functions. Answering this question is important, because p65RelA and p53/p63 have opposing effects on cell growth in mouse primary fibroblasts: p65RelA promotes cell proliferation and inhibits apoptosis (15–17), whereas p53/p63 induces apoptosis or senescence (1, 18, 19). In stratified epithelia, however, both p63 and p65RelA may work together to control epithelial stratification: p63 has been shown to be an upstream regulator of p65RelA, through its ability to induce IKKα expression (20). Furthermore, Wa3 mice, which spontaneously develop wavy hair, have been reported to carry a mutation in the genomic locus that encodes for the iASPP protein, and it has also been proposed that the observed phenotype is caused by a defect in the NFκB pathway (21). Hence, it is possible that the observed phenotype could be caused by the ability of iASPP to regulate p63's function upstream of p65RelA.

To better understand how iASPP functions in vivo, we generated an iASPP knockout mouse in which exon 8 was deleted from the *PPP1R13L* genetic locus (iASPP gene) using a Cre/loxP system. The ability of iASPP to affect cell growth was first tested using mouse embryonic fibroblasts (MEFs). The regulation of iASPP expression in response to differentiation stimuli was also

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[1]M.N. and Y.H. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: Xin.Lu@ludwig.ox.ac.uk.

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studied in mouse primary keratinocyte (KC) cultures. The biological significance of iASPP in regulating epithelial stratification in vivo was further investigated by examining the morphological differences between two stratified epithelial tissues, the epidermis and esophagus, of iASPP wild-type and null mice. The results reported here show that iASPP is an important regulator of p63 and is involved in controlling cell growth and epithelial stratification.

Results

iASPP Prevents Premature Cellular Senescence in Vitro. To understand the biological importance of iASPP in vivo, we generated a strain of transgenic mice in which iASPP expression was controlled by the bacterial Cre/loxP DNA recombination system. Mice carrying the heterozygous conditional iASPP allele ($iASPP^{flox/+}$), in which exon 8 of the PPP1R13L gene is surrounded by two loxP sites, were crossed with CMV-Cre^{+} transgenic mice (22) to generate $iASPP^{\Delta 8/+}$; Cre^{+} mice ($iASPP^{\Delta 8/+}$). The resulting heterozygous $iASPP^{\Delta 8/+}$ mice were used to produce iASPP homozygous mutant progeny, $iASPP^{\Delta 8/\Delta 8}$, in which exon 8 of the iASPP gene was eliminated. Deletion of exon 8 was confirmed by PCR analysis (Fig. S1*A*). Removal of exon 8 resulted in a shift of iASPP's ORF, and the absence of iASPP expression was confirmed in MEFs using two different anti-iASPP antibodies that were raised against different regions of iASPP as indicated (residues 1–240 or residues 459–639) in Fig. S1*B*.

The impact of iASPP loss was examined in $iASPP^{+/+}$, $iASPP^{\Delta 8/+}$, and $iASPP^{\Delta 8/\Delta 8}$ MEFs. Passage 3 MEFs were plated into 96-well plates at a density of 0.25×10^4 cells per well. Twenty-four, 48, and 72 h after plating, cells were incubated with BrdU for 2 h to label S-phase cells. The amount of incorporated BrdU in each well was quantified by measuring light emitted using an anti-BrdU secondary antibody conjugated with a chemiluminescent substrate. The lowest level of BrdU incorporation was detected in $iASPP^{\Delta 8/\Delta 8}$ MEFs, whereas the incorporation rate of BrdU in $iASPP^{\Delta 8/+}$ MEFs was intermediate compared with $iASPP^{+/+}$ and $iASPP^{\Delta 8/\Delta 8}$ MEFs (Fig. S1*C*). In late passage cells, $iASPP^{\Delta 8/\Delta 8}$ MEFs grew much slower than $iASPP^{+/+}$ MEFs, exhibited enlarged and flattened cell morphology, and stained positive for senescence-associated β-galactosidase (SA-β-gal), a well-known marker for cellular senescence (Fig. S1*D*). A small but detectable increase in $p19^{arf}$ and $p21^{waf1/CIP1}$ expression was also observed in passage 6 $iASPP^{\Delta 8/+}$ and $iASPP^{\Delta 8/\Delta 8}$ MEFs, agreeing with their ability to prematurely enter senescence (Fig. S1*D*). These data suggest that iASPP may play a role in preventing premature senescence in MEFs.

To investigate whether acute deletion of iASPP can cause premature cellular senescence, $iASPP^{flox/flox}$ mice were crossed with R26Cre^{+}-ER^{T} mice (23) to generate $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ mice in which Cre expression is controlled by the presence of 4-hydroxytamoxifen (4-OHT). $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs were generated and iASPP expression controlled by the presence or absence of 1 μM 4-OHT. Four days after the addition of 4-OHT, iASPP expression was undetectable (Fig. S2*A*). The ability of iASPP to affect proliferation was first analyzed by counting the numbers of viable cells using trypan blue staining, 24, 48, and 72 h after plating in the presence or absence of 4-OHT. In passage 2 MEFs, iASPP status did not affect cell growth. Reduced proliferation was initially observed using passage 3 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs in which iASPP was deleted by the presence of 4-OHT (Fig. S2*B*). A significant reduction in cell growth was subsequently observed in both passage 4 and passage 5 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs upon the addition of 4-OHT. Importantly, the presence or absence of 4-OHT did not affect the growth rate of $iASPP^{flox/flox}$; $Cre^{-}ER^{T}$ MEFs (Fig. 1*A*).

$iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs from different passages were also used to examine their ability to incorporate BrdU. Cells were grown in the presence or absence of 1 μM 4-OHT for 4 d and then incubated with BrdU for 2 or 24 h to label fast or slowly proliferating cells, respectively. Over 20% of passage 2 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs were labeled by a 2-h BrdU pulse, agreeing with the highly proliferative nature of this cell population. iASPP deletion in passage 3 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs caused only a small, but detectable, reduction in the number of $BrdU^{+}$ cells. Importantly, passage 4 and 5 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs, in which iASPP expression was deleted by the addition of 4-OHT, resulted in a 5- to 10-fold reduction in the number of $BrdU^{+}$ cells (from 15 to 3% or 10 to 0.1% for iASPP wild-type or null MEFs, respectively) after a 2-h BrdU pulse (Fig. S2*C*, bar graph). Similarly, loss of iASPP expression resulted in a 2- to 6-fold reduction in the number of slow cycling cells in passage 4 and 5 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs, labeled with BrdU for 24 h (from 65 to 30% or 60 to 10%, respectively) (Fig. 1*B*, bar graph). Finally,

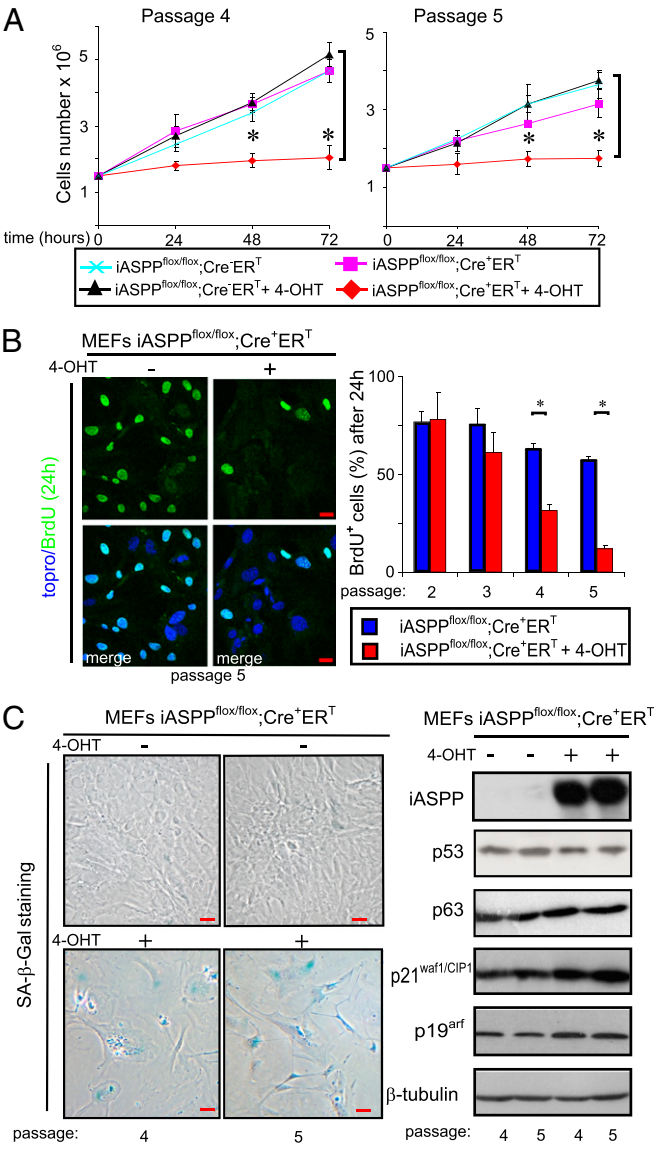

Fig. 1. iASPP deficiency induces premature cellular senescence. (*A*) Growth curve analyses of $iASPP^{flox/flox}$; $Cre^{\pm}ER^{T}$ with or without 4-OHT. Values for each time point represent the average ± standard deviation (SD) of three independent experiments. (*B*) $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs at different passages were incubated with BrdU for 24 h before immunofluorescence staining to visualize $BrdU^{+}$ cells. Bar graphs represent the percentage ± SD of $BrdU^{+}$ cells ($n = 3$) $^{*}P < 0.05$. (*C*) $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs were assayed for SA-β-gal activity with or without 4-OHT. Blue cells represent senescent cells. (Scale bar, 20 μm.) (*D*) Immunoblot analysis of iASPP, $p21^{waf1/CIP}$, $p19^{arf}$, p53, and p63 in passage 4 and 5 $iASPP^{flox/flox}$; $Cre^{+}ER^{T}$ MEFs. 4-OHT deletes exon 8 of the iASPP gene. β-tubulin was used as a loading control.

to investigate whether the dramatic reduction of S phase entry in iASPP-null iASPP$^{flox/flox}$; Cre^{+}ER^{T} MEFs was caused by premature senescence, we used SA-β-gal to stain senescent cells. As expected, SA-β-gal^{+} cells were predominantly observed in passages 4 and 5 iASPP-deficient iASPP$^{flox/flox}$; Cre^{+}ER^{T} MEFs (Fig. 1*C*). Quantification analyses revealed that using passage 4 and 5 iASPP-null MEFs, 45 and 65% of cells, respectively, were positive for SA-β-gal staining, compared with only 5% in iASPP-expressing MEFs (Fig. S2*D*, bar graph). Mechanistically, iASPP depletion did not affect p53 expression (Fig. 1*D*). However, a small but detectable increase in p19^{arf} and p21$^{waf1/CIP1}$ expression was observed in iASPP-deleted iASPP$^{flox/flox}$; Cre^{+}ER^{T} MEFs (Fig. 1*D*). These findings suggest that iASPP plays a key role in inhibiting premature cellular senescence.

iASPP Is a Negative Regulator of Keratinocyte Differentiation in Vitro. Mouse primary KCs are normally grown in low Ca^{2+}-containing medium (0.05 mM) to maintain their proliferative potential. An increase of extracellular Ca^{2+} concentration to 1.2 mM is able to induce nonconfluent KC cultures to differentiate in vitro, in a way that resembles in vivo epidermal stratification (24). To differentiate, KCs must inhibit the activity of factors that maintain their self-renewal potential and proliferative capacity. One of the most well-known transcription factors for epidermal self-renewal is p63 (7, 25). A reduction in p63 expression is accompanied by cell cycle exit and the appearance of differentiation markers such as keratins 1/10 or loricrin (26, 27). We, therefore, investigated whether iASPP plays a role in controlling KC differentiation, using mouse KCs purified from iASPP wild-type newborn mice maintained in low Ca^{2+}. Differentiation was induced by adding high Ca^{2+} (1.2 mM) medium for up to 5 d. KC differentiation was confirmed by a dramatic decrease in p63 expression and the translocation of E-cadherin to cell-to-cell junctions (26, 28) (Fig. 2*A*). Interestingly, we observed a significant reduction of iASPP expression in differentiated KCs cultured in high Ca^{2+}-containing medium for 5 d (Fig. 2*B*). This result suggests that iASPP may play a role in inhibiting KC differentiation in vitro.

To test this hypothesis, we generated mouse KCs from newborn iASPP$^{flox/flox}$; Cre^{+}ER^{T} mice. The expression of iASPP was deleted by adding 4-OHT to low Ca^{2+} media for 4 d, and the absence of iASPP was confirmed by immunoblot analysis (Fig. 2*C*). The ability of iASPP to affect KC differentiation was tested by incubating KC cultures in high Ca^{2+}-containing medium for 24 or 48 h, as indicated in Fig. 2*C*. The expression levels of differentiation markers such as keratin 1 (K1) and loricrin were compared between iASPP-expressing and -deficient mouse KCs. Interestingly, iASPP deficiency resulted in a small but detectable increase in the expression levels of K1 and loricrin. This increase in KC differentiation markers occurred as early as 24 h after the Ca^{2+} switch (Fig. 2*C*). Collectively, these results suggest that iASPP may negatively regulate KC differentiation.

iASPP Is Predominantly Expressed in the Basal Layer of Stratified Epithelia in Vivo. We observed that iASPP was mostly expressed in the basal layer of the epidermis (Fig. 3*A*). The specificity of the anti-iASPP antibody used (LX049.3) was confirmed by the lack of iASPP expression from iASPP$^{\Delta 8/\Delta 8}$ epidermis (Fig. 3*A*). Using double immunofluorescence staining, we observed that the majority of iASPP-expressing cells also expressed keratin 14 (K14), a well-known marker of basal epithelial cells with proliferative potential (27). Importantly, iASPP expression was mutually exclusive to K1 or loricrin, both markers that identify differentiated cells in the spinous and granular epidermal layers (27) (Fig. 3*B*).

Similarly, iASPP was abundantly expressed in the K14^{+} layer of mouse esophagus. Nuclear iASPP was occasionally detected in K14^{+} basal cells (Fig. S3*A*, arrowheads). Furthermore, iASPP and loricrin or iASPP and filaggrin expression were mutually exclusive as loricrin and filaggrin are expressed in differentiated suprabasal cells (29) (Fig. S3*A*). Together, these data provide important in vivo evidence that iASPP is expressed in basal epithelial cells, and its expression pattern agrees with its ability to maintain their proliferative potential and inhibit differentiation.

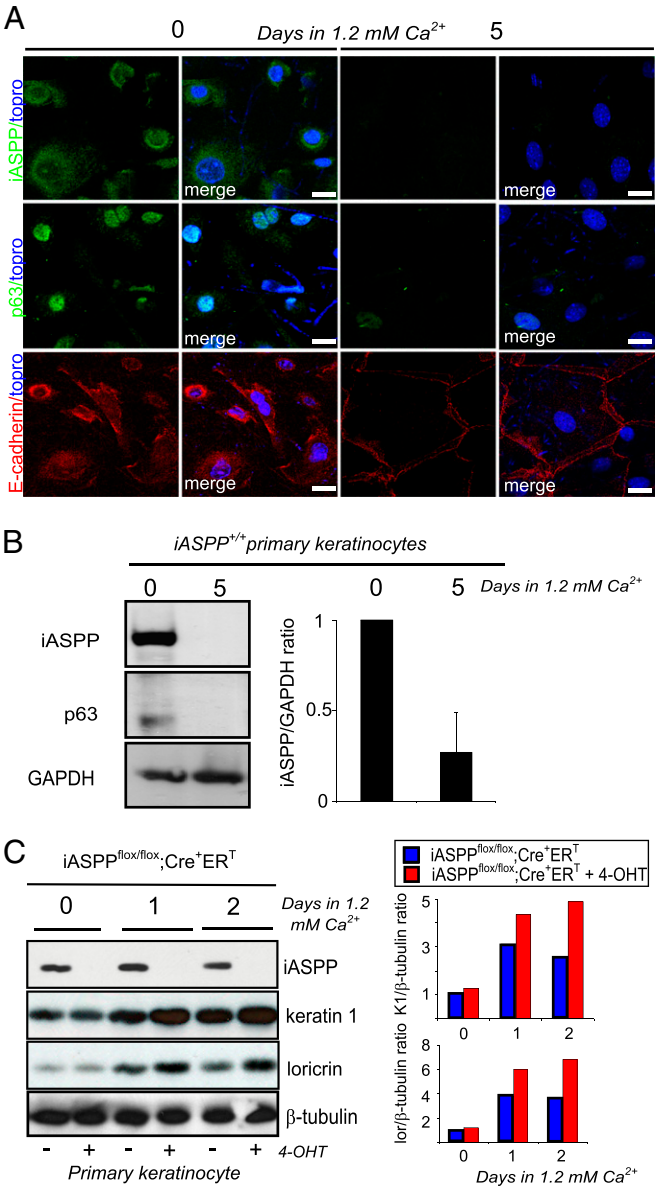

Fig. 2. iASPP inhibits Ca^{2+}-induced mouse primary KC differentiation. KCs were purified from newborn iASPP$^{+/+}$ mice, kept in high Ca^{2+} medium for the indicated time, and subjected to immunofluorescence (*A*) and immunoblotting (*B*) analyses to examine the expression pattern and levels of iASPP, p63, and E-cadherin. TO-PRO stains DNA. (Scale bar, 20 μm.) Bar graphs represent the ratio between iASPP and GAPDH expression, calculated using ImageJ densitometric analysis ($n = 4$). (*C*) iASPP$^{flox/flox}$; Cre^{+}ER^{T} KCs were treated with 4-OHT for 4 d to delete iASPP expression before exposure to high Ca^{2+} medium for the indicated time. Immunoblotting showed the expression levels of iASPP, keratin 1, and loricrin. β-tubulin expression was used as a loading control. Bar graphs represent the expression levels of *K1* and *loricrin* normalized against β-tubulin. The expression levels of *K1* and *loricrin* in iASPP$^{flox/flox}$;Cre^{+}ER^{T} KCs cultured under low Ca^{2+} conditions was set as 1. ($n = 2$).

iASPP Is an Important Regulator of Epithelial Stratification in Vivo. Histological analyses of wild-type and iASPP$^{\Delta 8/\Delta 8}$ epidermis revealed that loss of iASPP expression caused profound morphological abnormalities in epidermal structure associated with a noticeably thicker epidermis compared to the wild-type (Fig. 4*A*). Similarly, the stratified epithelia of iASPP mutant esophagus

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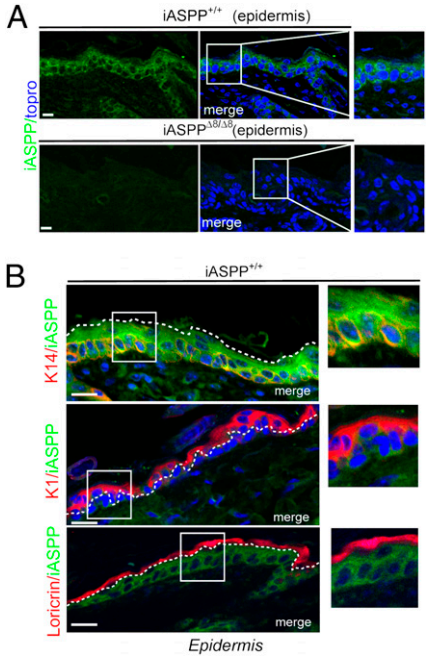

Fig. 3. iASPP is predominately expressed in the basal layer of the epidermis. (*A*) Immunofluorescence staining of mouse epidermis from iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice confirms the specificity of anti-iASPP antibody LX049.3. (*B*) Double immunofluorescence staining of iASPP and various epidermal markers. White dotted line represents the separation between iASPP and the epidermal markers. Squares represent higher magnification of the epidermal region. [Scale bar, 20 μm (*A*), 50 μm (*B*).]

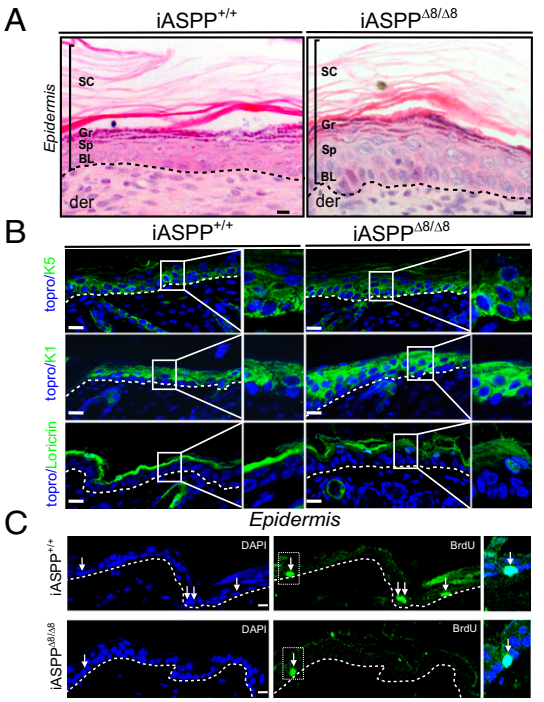

Fig. 4. Loss of iASPP impairs epithelial stratification in vivo. (*A*) H&E staining of iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ epidermal sections. BL, basal layer; Sp, spinous layer; Gr, stratum granulosum; SC, stratum corneum. Vertical bars represent epidermal thickness. (*B*) Immunofluorescence staining of various epidermal markers in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ epidermis. Squares represent higher magnifications of the areas in the picture. Dotted white line represents dermo–epidermal boundary. (Scale bar, 20 μm.) (*C*) Immunofluorescence staining of BrdU^{+} cells, indicated by white arrows, in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ epidermis. Dotted white line represents dermo–epidermal boundary. [Scale bars, 10 μm (*A*), 20 μm (*B*).]

exhibited an expansion of early differentiation layers (Fig. S4 *A* and *B*). Immunofluorescence staining of mouse iASPP$^{\Delta 8/\Delta 8}$ back epidermis with various epidermal markers demonstrated a clear expansion of differentiated layers (Fig. 4*B*). Expansion of K1- and loricrin-positive layers was evident in iASPP mutant specimens. Cell counts showed that although iASPP deficiency affected the number of K5/K14 expressing basal cells, its impact was not statistically significant (Fig. S4*C*, bar graph basal layer, $P = 0.1066$). However, iASPP deficiency did cause an expansion in the number of suprabasal KCs that expressed K1. The total number of cells in the epidermis was significantly increased in iASPP$^{\Delta 8/\Delta 8}$ mice when compared with their wild-type counterparts (Fig. S4*C*), indicating an expansion of the differentiated layers in the epidermis of iASPP transgenic mice. To examine the in vivo proliferation rate in iASPP$^{\Delta 8/\Delta 8}$ epidermis, the S-phase KCs were labeled by the thymidine analog BrdU, injected 3 h before the epidermis was dissected from wild-type and iASPP mutant animals. BrdU^{+}-labeled nuclei were confined to the basal layer of the epidermis (Fig. 4*C*). In iASPP$^{+/+}$ mice, around 4% of basal cells were BrdU labeled. However, the number of BrdU^{+} cells was reduced by 50% in iASPP mutant epidermis (Fig. 4*C*, bar graph). This result confirmed that iASPP deficiency reduces cell proliferation. The observed expansion of the suprabasal layer in iASPP mutant epidermis is likely to be caused by an increase in differentiation.

iASPP Binds and Colocalizes with p63 in Vivo and Antagonizes Its Transcriptional Activity. Knowing that iASPP is predominantly expressed in the basal layers of stratified epithelia in mouse skin and esophagus, we investigated whether a similar expression pattern exists in humans. Immunofluorescence analysis of normal human esophagus showed that iASPP was mainly expressed as a nuclear protein in the basal layers of stratified epithelia; however, its expression was more cytoplasmic in the suprabasal layers (Fig S5*A*). Using adjacent sections from human cervix, we also observed the nuclear location of iASPP in the basal layer, and a more cytoplasmic location of the protein in the differentiated suprabasal layers (Fig. 5*A*). Moreover, nuclear iASPP overlapped with that of p63 in human cervical epithelia (Fig. 5*A*). As iASPP has been identified to be a binding partner of p63 and p65RelA, we also carried out double immunofluorescence staining to examine the expression patterns of iASPP/p63 or iASPP/p65RelA in mouse esophagus and human epidermis. Interestingly, in mouse esophagus, nuclear iASPP was predominantly detected in p63-expressing basal KCs (Fig. S5*B*, white arrows), despite a low frequency. In contrast, iASPP and p65RelA were almost mutually exclusive as p65RelA was predominately expressed in the suprabasal layers (Fig. S5*B*). Similar expression patterns of iASPP/p63 and iASPP/p65RelA were observed in human epidermis (Fig. 5*B*) where, under the same conditions, p65RelA was predominantly expressed as a cytoplasmic protein in upper differentiated layers, whereas iASPP had nuclear and cytoplasmic locations in basal and suprabasal KCs, respectively. Nuclear iASPP colocalized with p63 in basal KCs but rarely colocalized with p65RelA (Fig. 5*B*, white arrows). The observed colocalization of nuclear iASPP and p63, but not p65RelA, in basal cells suggests that iASPP is likely to influence epithelial stratification through its ability to regulate the transcriptional activity of p63, rather than p65RelA.

Because the DNA binding domain of p63 and C terminus of iASPP interact in vitro (13), we hypothesized that iASPP may bind and antagonize p63's function in controlling epithelial stratification. Hence, we examined the capacity of iASPP to interact with p63 in the human KC cell line HaCat. As an interaction between endogenous iASPP and p63 was detected (Fig. 5*C*), the ability of iASPP to influence the transcriptional activity of p63 was then tested. Established transcriptional targets of TAp63 and ΔNp63 are envoplakin (30) and K14 (31), re-

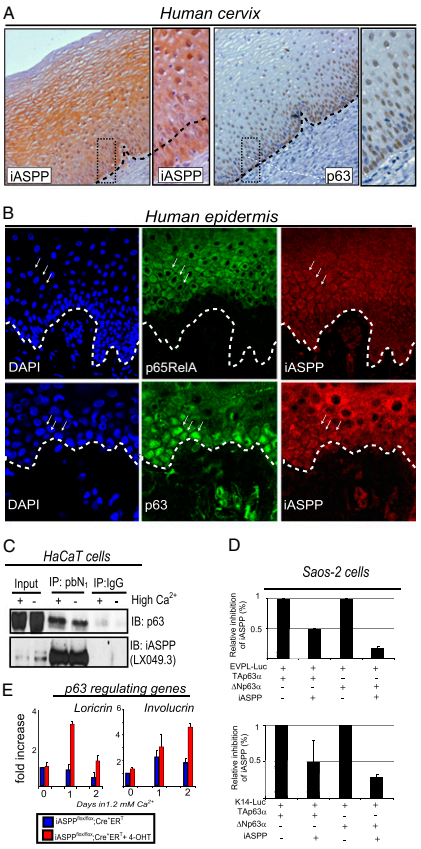

Fig. 5. iASPP binds and colocalizes with p63 and inhibits its transcriptional activity. (*A*) Immunohistochemical staining of iASPP (LX049.3) and p63 (4A4) using adjacent human cervical sections shows that iASPP and p63 are expressed in the nuclei of basal epithelial cells. Dotted line represents dermo–epidermal boundary. Squares represent higher magnifications of the areas in the picture. (*B*) Double immunofluorescence staining of iASPP and p65RelA *(Upper)* or p63 *(Lower)* in human epidermis. Nuclear iASPP is marked by white arrows. [Scale bar, 20 μm (*Upper*) and 40 μm (*Lower*).] Dotted line represents the dermo–epidermal boundary. (*C*) Coimmunoprecipitation of iASPP and p63 in HaCaT cells exposed to high Ca^{2+} for 24 h using anti-iASPP and anti-p63 antibodies as indicated. (*D*) Bar graph shows the effect of iASPP on the transcriptional activity of TAp63α or ΔNp63 on the *envoplakin* (EVLP-Luc) (*Left*) or *K14* (K14-Luc) (*Right*) promoters, as indicated. Values are the mean ± SD of three independent experiments ($n = 3$). (*E*) Quantitative real-time PCR measured the mRNA levels of indicated p63-regulated genes in the presence or absence of iASPP. Bar graphs represent the mRNA levels of target genes normalized against GAPDH. The mRNA levels of these target genes in iASPP wild-type KCs cultured under low Ca^{2+} conditions were set as 1. The data presented are the mean ± SD of three independent experiments ($n = 3$).

spectively. The envoplakin or K14 promoter linked to the luciferase reporter gene (EVPL-Luc) or (K14-Luc) was used as readout for the transcriptional activity of TAp63α or ΔNp63α, respectively. To investigate the ability of iASPP to influence the transcriptional activity of p63, the p53-null cell line Saos-2 was transfected with plasmids that express TAp63α or ΔNp63α, in the presence or absence of exogenous iASPP. As expected, TAp63α was more active in enhancing the transcriptional activity of EVPL-Luc than K14-Luc, whereas ΔNp63α was more active in transactivating K14-Luc than TAp63α (Fig. S5*C*). When the transcriptional activity of TAp63 or ΔNp63 was set as 1, coexpression of iASPP with TAp63α or ΔNp63α reduced their transcriptional activity by 50% compared with cells transfected with TAp63α or ΔNp63α alone (Fig. 5*D*). This decrease in transcriptional activity was not due to a reduction in TAp63α or ΔNp63α expression, as similar amounts of TAp63α or ΔNp63α were detected, irrespective of iASPP expression (Fig. S5*C*). Similar results were also observed in another p53-null cell line, H1299 (Fig. S5*D*). These results suggest that one of the mechanisms by which iASPP may affect epithelial stratification and cell cycle withdrawal is through its ability to bind p63 and inhibit its transcriptional activity.

Next, the ability of iASPP to influence the transcriptional activity of p63 on p63-regulating genes was next investigated using quantitative PCR: *K1, K10, K14, involucrin, loricrin* and *envoplakin* were tested. We also included a number of known p65RelA-regulating genes such as *TRAF-2, c-IAP1,* and *c-IAP2*. Four days after the addition of 4-OHT to deplete iASPP, the cells were switched from low to high Ca^{2+} medium to induce differentiation. The expression levels of the tested p63- or p65RelA-regulating genes in iASPP wild-type KCs, grown in low Ca^{2+} medium, were set as 1. The expression levels of these genes were then compared between iASPP wild-type and mutant cells, before or after they were switched to high Ca^{2+} medium. Interestingly, we observed a small increase in K1 expression in iASPP-deficient KCs compared with wild-type KCs, indicative of increased differentiation in iASPP-null KCs. Under the same conditions, iASPP status had very little impact on the expression levels of other p63- or p65RelA-regulating genes (Fig. S5*E*). One day after switching to high Ca^{2+} medium, there was a clear increase in the expression levels of K10, involucrin and envoplakin in both wild-type and $iASPP^{\Delta 8/\Delta 8}$ KCs, indicating that the cells were undergoing differentiation. Deletion of iASPP resulted in a small but detectable reduction in K14 expression on the one hand, and a minimal increase in K1, involucrin and envoplakin mRNA levels on the other. Interestingly, however, loricrin mRNA levels were more than threefold greater in iASPP-deficient KCs compared with iASPP wild-type cells (Fig. 5*E*). This agrees with the results in Fig. 2*C*, in which more loricrin was detected in iASPP-deficient KCs than in iASPP wild-type cells. Two days after switching to high Ca^{2+} medium, iASPP deficiency had a much smaller impact on the expression levels of loricrin under these conditions. Interestingly, involucrin mRNA was increased by over twofold in iASPP-deficient KCs in comparison with iASPP wild-type cells (Fig. 5*E*). Under the same conditions, iASPP deficiency had a minimal impact on p65RelA-regulating genes (Fig. S5*E*). These results demonstrate that iASPP deficiency promotes KC differentiation, and that the expression levels of loricrin and involucrin are most affected by iASPP status.

Discussion

iASPP is an inhibitor of p53-mediated apoptosis, and the most evolutionarily conserved member of the ASPP family (3). Using iASPP wild-type and null MEFs as an in vitro system, we have obtained important genetic evidence that iASPP has functions other than inhibiting apoptosis: i.e., loss of iASPP accelerates premature cellular senescence and KC differentiation. Because elevated p65RelA activity is known to increase cell proliferation (15), the acceleration of premature cellular senescence observed in $iASPP^{\Delta 8/\Delta 8}$ MEFs suggests that iASPP is unlikely to function as an inhibitor of p65RelA in mouse primary fibroblasts. In contrast, iASPP is likely to be an inhibitor of p53 or p63, as elevated activity of either protein induces cellular senescence (1, 18, 19). This is supported by the fact that loss of iASPP expression is often associated with small but detectable increases in $p21^{waf1/CIP1}$ and $p19^{arf}$ expression. Although we failed to observe any obvious changes in p53 or p63 protein levels, iASPP deficiency may enhance p53 and p63's transcriptional activity without affecting their expression, because iASPP is able to bind and inhibit the transcriptional activity of both. As both p53 and p63 can induce cellular senescence in MEFs (19, 32), this inhibitory property of iASPP may underlie its ability to prevent cellular senescence.

Using mouse primary KCs and epidermis from $iASPP^{+/+}$ and $iASPP^{\Delta 8/\Delta 8}$ mice, we also identified an important role for iASPP in inhibiting epidermal differentiation both in vivo and in vitro. This is predominately achieved through iASPP's ability to bind,

colocalize, and inhibit p63, a master transcription factor that is required to maintain the self-renewal and proliferative potential of the epidermis. At least six different p63 isoforms have been identified to date, which are divided into two main groups: transactivation domain-containing TAp63 versus N-terminal transactivation domain-deleted ΔNp63 (33). In theory, ΔNp63 could act as a dominant negative regulator of TAp63 in the basal layer of the epidermis (25). However, it is known that ΔNp63 isoforms can activate the transcription of target genes through a second transactivation domain (18, 34). Furthermore, both TAp63α and ΔNp63 can directly or indirectly control the expression of genes that contribute to epidermal differentiation (20, 30, 31). In the basal layer of stratified epithelia, either in the epidermis or in the esophagus, the predominant p63 isoform is ΔNp63 (35). Recently, it was reported that TAp63 also plays a key role in the maintenance of adult stem cells in murine skin (36). Hence both TAp63 and ΔNp63 can control epithelial stratification. Cellular factors like iASPP, which can bind both TAp63 and ΔNp63, are therefore likely to influence epithelial stratification by affecting the activity of both proteins. Further studies are needed to elucidate whether iASPP predominantly functions through either TAp63, ΔNp63, or both to regulate epithelial stratification.

Finally, in a previous report on Wa3 mice, it was proposed that iASPP may maintain epithelial homeostasis by binding and inhibiting the activity of p65RelA (21). Here, we have shown that iASPP can also affect epithelial stratification by binding p63 and inhibiting its transcriptional activity. This was further supported by the in vivo finding that nuclear iASPP and p63 predominantly colocalized in the basal layer of stratified epithelia, a region in which p65RelA is almost undetectable. In differentiated suprabasal KCs, p65RelA was predominately expressed as a cytoplasmic protein and rarely colocalized with iASPP in the nucleus. The fact that both p63 and p65RelA are transcription factors and need to be in the nucleus to perform their transcriptional activities, and iASPP only colocalized with p63 and not p65RelA in the nuclei of basal epithelial cells, supports the notion that iASPP is more likely to control epithelial stratification via p63 than p65RelA. Although IKKα is a direct transcriptional target of p63 (20), and IKKα is an upstream activator of p65RelA, IKKα's kinase activity is not required for it to regulate epithelial stratification (37). Hence, it is possible that iASPP may influence stratification via the p63/IKKα pathway, independently of p65RelA. Future genetic studies are needed to test this hypothesis. Nonetheless, this study identifies iASPP as a key player in epithelial stratification, a function that is achieved through its ability to bind and inhibit p63's activities and suppress cellular senescence and terminal differentiation.

Materials and Methods

iASPP$^{flox/+}$ mice were generated by InGenious Targeting Laboratory in a mixed C57BL/6Jx129vJ background. Genotyping of iASPP$^{\Delta 8/\Delta 8}$ mice was performed by PCR analysis using primers FLP2 (5′-CCGAATTGGAGAAGTGAAGC-3′), I8-2 (5′-CCGAATTGGAGAAGTGAAGC-3′), and E8-2 (5′-AGAGCAGCCTCAGAGCATGG-3′). iASPP$^{flox/+}$ conditional mice were crossed with R26Cre^{+}-ER^{T} mice (22) to induce deletion of exon 8 upon treatment with 4-OHT (Sigma-Aldrich). All animal procedures were approved by local ethical review and licensed by the U.K. Home Office. For more details, see *SI Materials and Methods*.

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