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iASPP/p63 autoregulatory feedback loop is required for the homeostasis of stratified epithelia

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iASPP, an inhibitory member of the ASPP (apoptosis stimulating protein of p53) family, is an evolutionarily conserved inhibitor of p53 which is frequently upregulated in human cancers. However, little is known about the role of iASPP under physiological conditions. Here, we report that iASPP is a critical regulator of epithelial development. We demonstrate a novel autoregulatory feedback loop which controls crucial physiological activities by linking iASPP to p63, via two previously unreported microRNAs, miR-574-3p and miR-720. By investigating its function in stratified epithelia, we show that iASPP participates in the p63-mediated epithelial integrity program by regulating the expression of genes essential for cell adhesion. Silencing of iASPP in keratinocytes by RNA interference promotes and accelerates a differentiation pathway, which also affects and slowdown cellular proliferation. Taken together, these data reveal iASPP as a key regulator of epithelial homeostasis.

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Introduction

The epidermis is a stratified, self-renewing epithelium composed of keratinocytes that are continuously regenerated by the terminal differentiation pathway. Proliferative keratinocytes located in the basal layer periodically withdraw from the cell cycle, migrate upwards and commit to differentiation before ultimately being shed from the skin surface (Watt, 1989; Fuchs, 1990). During the differentiation process, epidermal keratinocytes progress through several stages, with the resulting layered architecture forming a barrier to protect against infection, dehydration and mechanical stress. As basal layer keratinocytes differentiate, cell junctional components are integrated in a polarized fashion (Green and Gaudry, 2000; Green *et al*, 2010), with adherens junctions most enriched in basal keratinocytes and expression of desmosomal components changing during the stratification process (Delva *et al*, 2009). In contrast, a tight junction barrier is sealed specifically within the upper layer of granular cells before these give rise to the stratum corneum, a dead cell layer which serves as a scaffold for the deposition of a lipid bilayer. Thus, in the epidermis, adhesion exerts a dynamic role in epidermal differentiation and stratification mediated by hemidesmosomes and focal adhesions (which function in cell–matrix adhesion), and desmosomes, adherens and tight junctions (which function in cell–cell adhesion).

The ASPP (apoptosis stimulating protein of p53) proteins are a group of p53 co-activators (Trigiante and Lu, 2006). The apoptotic function of p53 is potentiated by ASPP1 and ASPP2, while a third family member iASPP, negatively modulates apoptosis. Inhibitory member of the ASPP (iASPP; encoded by *PPP1R13L*) is evolutionarily conserved from worm to human, and its expression is upregulated in human cancers (Bergamaschi *et al*, 2003, 2006; Zhang *et al*, 2005; Saebo *et al*, 2006). iASPP is expressed predominantly in epithelial cells, in the skin, testis, heart and stomach (Herron *et al*, 2005). Mutations in *Ppp1r13l* cause abnormalities of the heart and skin in both mice and cattle (Herron *et al*, 2005; Simpson *et al*, 2009). Mice which harbour a deletion mutation in *PPP1R13L* display wavy hair, open eyelids at birth and develop a rapidly progressive cardiomyopathy (Herron *et al*, 2005) while cattle which harbour a frame-shift mutation in bovine *PPP1R13L* exhibit cardiomyopathy and a woolly coat (Simpson *et al*, 2009). These changes are phenotypically similar to the human cardiocutaneous syndrome which is also characterized by cardiomyopathy, woolly hair and palmoplantar keratoderma (Protonotarios and Tsatsopoulou, 2004).

Structural studies with p53 family members have recently shown that iASPP preferentially binds to p63, a homologue of p53 (Robinson *et al*, 2008), which plays a crucial role in epithelial development (Mills *et al*, 1999; Yang *et al*, 1999). *TP63* is tissue specifically transcribed with two alternative promoters giving rise to TAp63 and ΔNp63 isoforms (Yang *et al*, 1998). During mouse embryonic development, TAp63 is expressed at the surface of the ectoderm prior to stratification and a shift in balance towards the truncated variant ΔNp63 is required for epidermal maturation (Koster *et al*, 2004; McKeon, 2004). In the mature epidermis, ΔNp63 is restricted to proliferative basal epidermal cells and is

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downregulated in more differentiated layers (Koster *et al*, 2004). Importantly, ΔNp63 maintains the stem cell population in the proliferative compartment of stratified epithelia (Mills *et al*, 1999; Yang *et al*, 1999; Pellegrini *et al*, 2001; Koster *et al*, 2005). Recent studies through conditional gene deletion show that p63 also regulates the proliferative potential of epidermal stem cells in adult skin and subsequently influences cell senescence and ageing in mice (Keyes *et al*, 2005; Senoo *et al*, 2007; Guo *et al*, 2009).

The molecular mechanism underlying the regulation of iASPP is still poorly understood. Using mouse and human skin cultures, we report a determinant mechanism linking iASPP and p63 through the participation of two unreported microRNAs, which act as negative regulators of p63 protein. This controls the epithelial integrity program, affecting cell adhesion, proliferation and differentiation. Taken together, these findings uncover an essential role of iASPP for epithelial homeostasis.

Results

iASPP expression in skin development

Detection of iASPP expression in mouse skin has previously been reported (Herron *et al*, 2005) although a functional role for iASPP in human skin has not yet been explored. In order to investigate a possible role for iASPP *in vivo*, the expression pattern of iASPP during mouse embryogenesis was established. Because p63 is the first keratinocyte-specific marker expressed in cells developing along a keratinocyte lineage pathway (Green *et al*, 2003), we compared expression of iASPP in relation to expression of p63 during epidermal morphogenesis. Immunohistochemical analysis (Figure 1A) showed iASPP expression in the developing epidermis of the early embryo (E12.5) which consists of two cell layers derived from the ectoderm, where p63 has initiated the epithelial stratification program (Koster and Roop, 2004). iASPP epithelial expression pattern is also maintained after commitment to differentiation (E15.5) where iASPP was expressed in the cytoplasm and demonstrated colocalization with p63 in the nuclei. These initial findings support the involvement of iASPP in the epidermal differentiation program.

We next investigated whether iASPP was expressed in the epidermal stem cell compartment. Keratinocytes were isolated from adult mice between 7 and 8 weeks of age. The basal cell population of the skin was sorted by high CD49F and the bulge stem cell population by high CD49F and high CD34 expression (Trempus *et al*, 2003). qRT–PCR data showed that *Ppp1r13l* was expressed in the bulge stem cell compartment at a similar level to CD49F-positive basal keratinocytes (Figure 1B). Analysis of the constituent cell compartments of normal human skin demonstrated PPP1R13L expression in fibroblasts, keratinocytes and melanocytes, with the highest mRNA abundance in keratinocytes (Figure 1C). We observed colocalization of iASPP with p63 in the nuclei of the basal and suprabasal layers of human epidermis (Figure 1D).

To investigate the physiological role of iASPP, we examined levels of endogenous iASPP in human primary keratinocytes and HaCaT cells (immortalized human keratinocytes) during differentiation. Cultured keratinocytes were able to differentiate *ex vivo* at a high calcium concentration confirmed by expression of specific keratinocyte differentiation markers including keratin 10 (K10) and concurrent downregulation of basal markers such as keratin 14 (K14) (Figure 1E). During keratinocyte differentiation, ΔNp63α levels decreased in both primary keratinocytes and HaCaT cells with a concomitant decrease observed in iASPP levels. These data were consistent with our data above, demonstrating the colocalization of iASPP and p63 expression in human skin. Thus, iASPP appears to be involved in the epidermal differentiation program.

iASPP and p63 are linked in an autoregulatory feedback loop

To determine the contribution of iASPP to regulation of epidermal formation, its interaction with p63 was explored. The human iASPP promoter contains three putative p53 binding sites that could be recognized by p63 (Supplementary Figure S1A). To establish whether p63 is a direct transcriptional regulator of *PPP1R13L*, chromatin immunoprecipitation (ChIP) was performed using HaCaT cells which endogenously express high levels of ΔNp63 (Figure 2A). Immunoprecipitation with specific anti-p63 antibodies (detecting p63 C′ and N′ terminus, respectively) demonstrated that p63 binds to the *PPP1R13L* promoter *in vivo*. To confirm that p63 expression affects iASPP protein expression, HEK293 cells (Human Embryonic Kidney cells, with undetectable p63 and low iASPP levels) were transiently transfected with TAp63 and ΔNp63 isoforms. Increased levels of endogenous iASPP expression were observed at both the protein and mRNA levels (Figure 2B; Supplementary Figure S1B). Moreover, depletion of p63 by small interfering RNA (siRNA) in keratinocytes significantly reduced endogenous iASPP protein and mRNA expression without affecting p53 (Figure 2C and D). Although at the protein level TAp63 is almost undetectable at the RNA level iASPP is clearly downregulated by both TA and ΔNp63 siRNA (Figure 2D; Supplementary Figure S1C). Similarly, upregulation of iASPP in HEK293 cells reactivates p63 expression (Figure 2E; Supplementary Figure S1D), while silencing of *PPP1R13L* by siRNA in primary keratinocytes as well as in a range of keratinocyte cell cultures drastically decreased ΔNp63 and TAp63 protein expression independently of p53 and without affecting IRF6, recently reported as having a regulatory feedback loop with ΔNp63 (Moretti *et al*, 2010; Figure 2F; Supplementary Figure S1E and F). To further assess the reciprocal regulation between iASPP and p63, in a more physiological context, primary keratinocytes were treated with UV-B and both iASPP and p63 proteins were similarly downregulated at 25 mJ/cm^2 (Figure 2G; Supplementary Figure S1G). However, depletion of iASPP failed to alter *TP63* mRNA expression, suggesting modulation of the protein by an intermediary mechanism (Figure 2H; Supplementary Figure S1H). Taken together, these findings suggest that iASPP and p63 (both ΔN and TA) are linked in an autoregulatory feedback loop that is not influenced by p53 expression.

iASPP controls p63 through microRNA regulation

To explore the regulation of p63 by iASPP in human keratinocytes, a retroviral vector expressing short-hairpin RNA (shRNA) was used to stably knock down iASPP protein levels in keratinocytes. One unique shRNA construct specifically

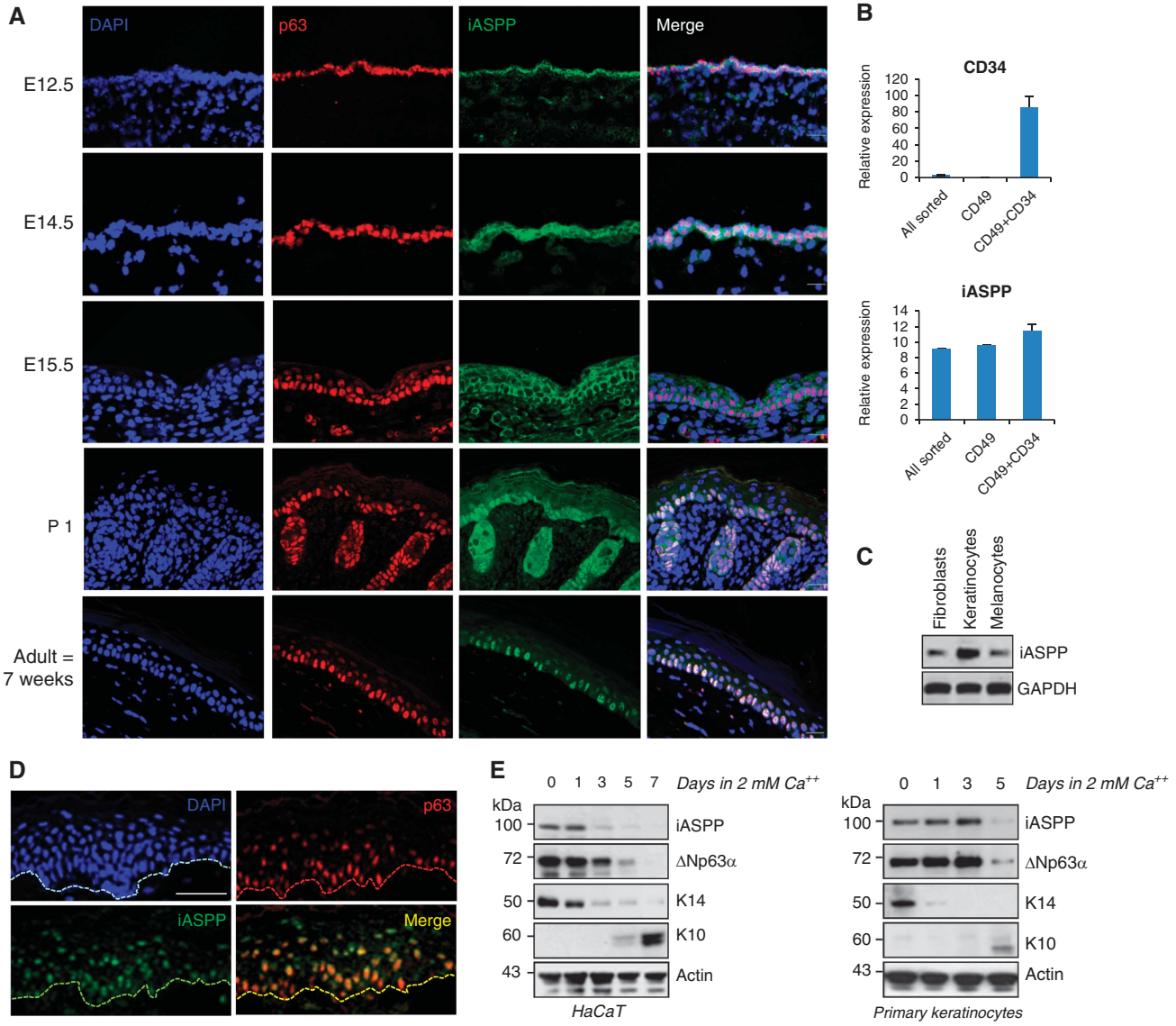

Figure 1 iASPP expression in skin development. (**A**) Observation of iASPP expression in developing skin during embryogenesis by immunofluorescence. Indirect immunofluorescence microscopy of skin sections taken from mice embryo during development ranging from 12.5 days to adult mice (7 weeks) revealed co-expression and colocalization of iASPP and p63. DAPI (blue) is used as a nuclear stain. Scale bar: 100 μm. (**B**) Detection of iASPP mRNA in the bulge stem cell population performed by quantitative PCR. Freshly isolated mouse keratinocytes were FACS sorted for the bulge stem cell population ($CD34^{high}CD49^{high}$), the basal cell population ($CD49^{high}$) and all sorted. Analysis of CD34 expression confirms correct and clean sorting of the respective populations. Equivalent expression of iASPP expression was observed in the basal and bulge stem cell populations. Relative expression was determined by normalization with the β-actin housekeeping gene. (**C**) Observation of iASPP mRNA expression in cellular components of human epidermis. Semiquantitative RT–PCR reveals high expression of iASPP in keratinocytes. GAPDH is shown as a loading control. (**D**) iASPP localization in human adult skin. Immunofluorescence microscopy shows iASPP expression in the nuclei of the basal and spinous layers of the epidermis. Control staining with p63 (basal epidermal layer marker) confirms colocalization with iASPP in this compartment of the epidermis. Scale bar: 50 μm. (**E**) Induction of differentiation in keratinocytes using high serum calcium results in decrease of iASPP and p63 proteins, as shown by western blot analysis in both HaCaT cells and primary keratinocytes at the time points indicated. K14 and K10 were used as markers to confirm proliferation and differentiation, respectively. Actin is shown as a loading control.

targeted and reduced endogenous iASPP at both protein and mRNA levels (Figure 3A and B). In keeping with earlier findings reported using siRNA-iASPP, *TP63* mRNA expression was not affected in the shRNA-iASPP cells while p63 protein levels were significantly reduced. Treatment with a specific inhibitor of proteasome-mediated degradation, MG132, was not sufficient to restore ΔNp63 protein levels in iASPP-silenced keratinocytes indicating that iASPP does not affect p63 stability (Figure 3C). Therefore, in order to establish whether modulation of p63 by iASPP could alternatively occur via microRNAs (miRNA), an Agilent MicroRNA Profiling assay was performed. Three miRNAs were found to be upregulated as a consequence of iASPP silencing (Figure 3D). Target prediction, based on implementation of the miRanda algorithm, revealed that two of these three induced miRNAs, hsa-miR-574-3p and hsa-miR-720, were likely to control p63. Increased specific expression of the two identified miRNAs in the iASPP knockdown cells was demonstrated when compared with the control scrambled shRNA-treated cells or versus a miRNA (miR-193a-3p)

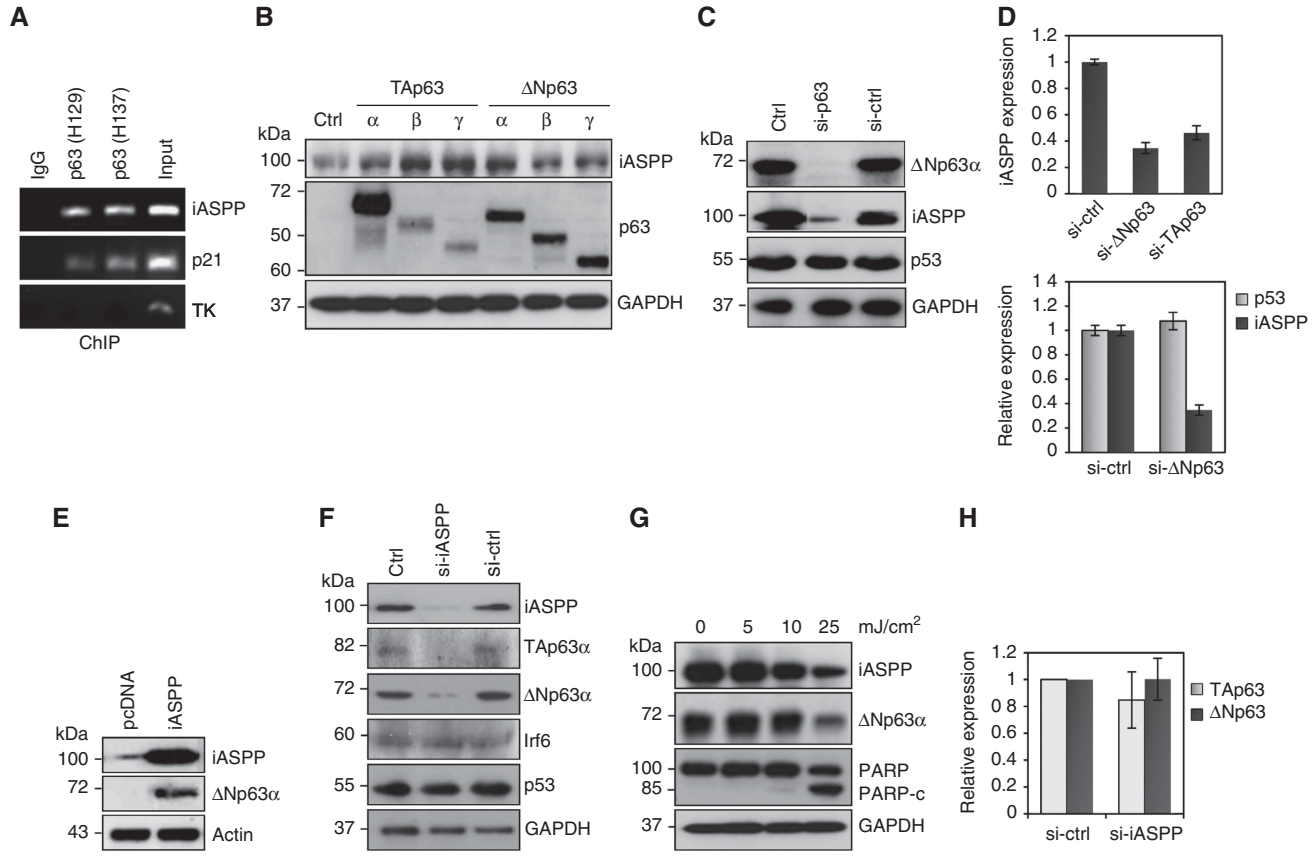

Figure 2 iASPP/p63 autoregulatory feedback loop. (**A**) ChIP assay showing binding of both the C′ and N′ terminus of p63 protein to iASPP promoter. Lane 1: isotope control antibody; lane 2: anti-p63 antibody H129 detecting the C′ terminus of p63; lane 3: anti-p63 antibody (H137) detecting the N′ terminus of p63; lane 4: input. Thymidine kinase (TK) promoter was used as a negative control while p21 was used as a positive control. (**B**) Western blots showing overexpression of p63 isoforms in HEK293 cells. All isoforms of TAp63 and ΔNp63 induce expression of iASPP at the protein level. GAPDH is shown as a loading control. (**C**) Western blot analysis showing depletion of p63 by siRNA in primary keratinocytes reflected in a concomitant downregulation of iASPP compared with control and siRNA-scramble (si-ctrl) cultures. (**D**) Upper panel: qRT–PCR performed on primary keratinocytes transfected with siRNA-scramble (si-ctrl), siRNA-ΔNp63 and siRNA-TAp63 showing the relative expression of iASPP mRNA. The lower panel corresponds at the qRT–PCR for the relative expression of p53 mRNA in the primary keratinocytes transfected with siRNA-scramble (si-ctrl) and siRNA-ΔNp63. (**E**) Western blot analysis of HEK293 cells overexpressing iASPP causes induction of ΔNp63. (**F**) Western blot analysis showing how depletion of iASPP by siRNA efficiently downregulates p63 protein levels in primary keratinocytes cells compared with control cultures and siRNA-scramble (si-ctrl). (**G**) Western blot analysis for p63 and iASPP expression in UV-B-irradiated primary keratinocytes with 5, 10, 25 and 50 mJ/cm^2 of UV-B. GAPDH was used as an internal control. (**H**) qRT–PCR performed on primary keratinocytes transfected with siRNA-scramble (si-ctrl), and siRNA-iASPP reveals no statistical significant variation in relative expression of TA and ΔNp63 mRNA.

unaffected by iASPP silencing, confirming the microRNA array data (Figure 3E). The efficiency of the specific antagomirs was also assessed by expression of them in the sh-iASPP-silenced cells. When miR-720 and miR-574-3p were, respectively, co-expressed with a luciferase reporter gene containing the 3′UTR of human p63, significant reduction of luciferase activity was observed in HEK293 cells (Figure 3G). Transduction only of specific antagomirs for miR-574-3p and miR-720 in keratinocytes restored ΔNp63 endogenous protein levels in sh-iASPP cells while an antagomir against miR-193a-3p fail to do so (Figure 3F). Furthermore, when primary keratinocytes are cultured in high calcium concentrations, increased expression of differentiation markers such as involucrin correlates with progressive upregulation of miR-720 and miR-574-3p (Figure 3H) while both anti-miR-720 and anti-miR-574-3p prevent the downregulation of ΔNp63 typically observed during differentiation (Figure 3I). Depletion of iASPP does not affect *TP63* mRNA levels, suggesting that both miRNAs are acting through inhibition of p63 translation. Taken together, these data demonstrate that iASPP represses miR-720 and miR-574-3p which in turn could negatively regulate p63, providing a further mechanism through which iASPP can regulate p63 in the skin independently of p53. Moreover, this is the first evidence of two novel non-coding RNAs that control expression of p63 isoforms during skin differentiation.

iASPP regulates epidermal adhesion and proliferation

Microarray analysis was used to determine the function of iASPP in keratinocytes. The biological processes over-represented within the list of genes modulated by *PPP1R13L* silencing (Supplementary Figure S2) are detailed in Supplementary Table S1. CD47, a plasma membrane protein, physically and functionally associated with integrins (Porter and Hogg, 1998) was significantly downregulated by iASPP depletion (Supplementary Figure S3A). Recent studies have highlighted the involvement of p63 proteins in cell adhesion, including integrin-mediated cell adhesion

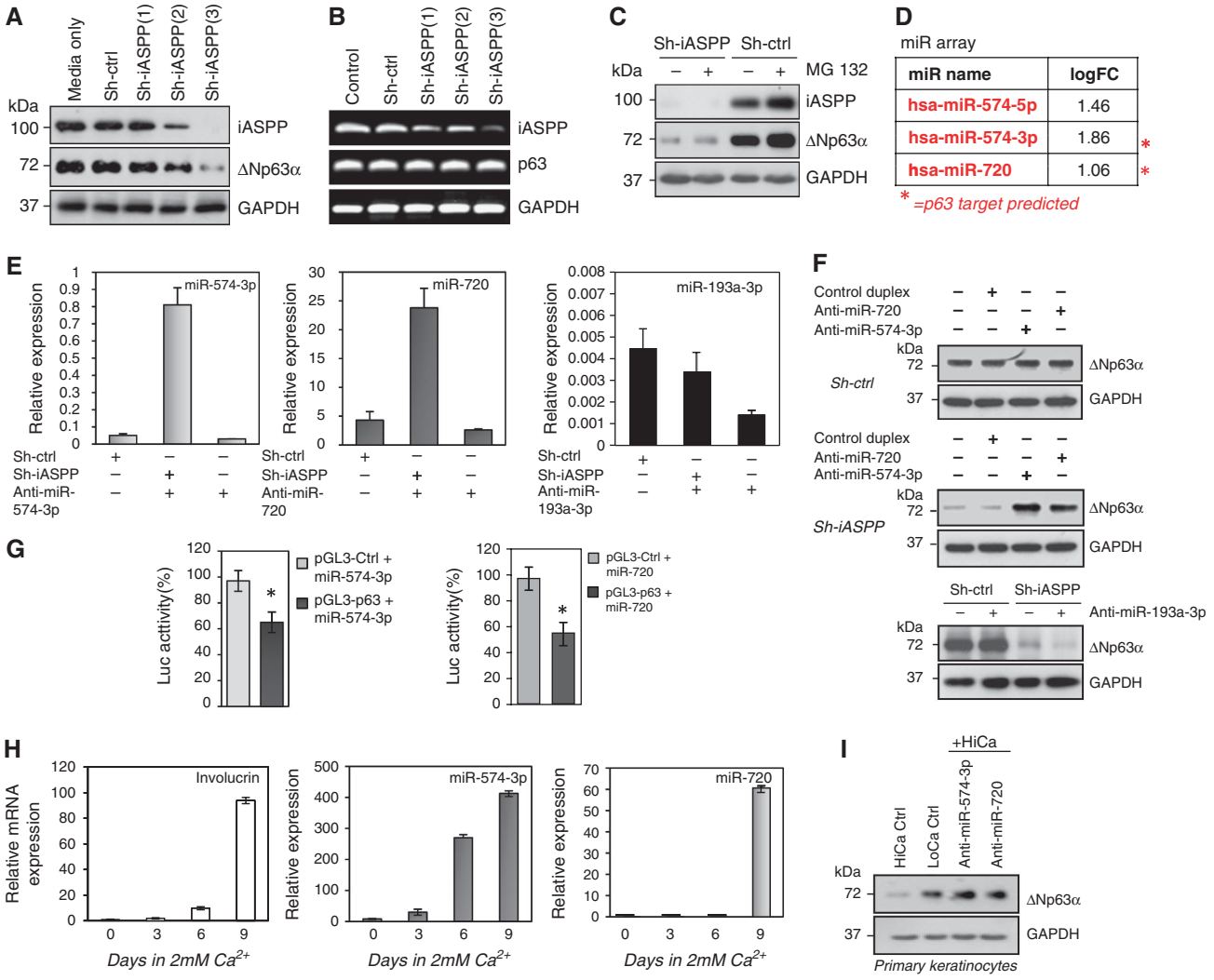

Figure 3 iASPP controls p63 through miRNA regulation. (**A**) Western blot analysis of several short-hairpin RNAs (shRNAs) that target *iASPP* gene in HaCaT cells. Construct 3 provides the best knockdown of iASPP protein and will be used from now on in the rest of the manuscript. (**B**) RT–PCR confirming specific targeting of iASPP sequence by shRNA. iASPP silencing fails again to inhibit mRNA levels of p63 in HaCaT cells. (**C**) Western blot analysis of iASPP-silenced keratinocytes treated with the proteosome inhibitor MG132 (5 mM, 6 h). Block of the proteosomal degradation is not sufficient to restore iASPP and ΔNp63 protein expression. (**D**) Log-fold change values of three miRNAs showing greatest overexpression in shRNA-iASPP versus shRNA-scramble HaCaT cells in the microRNA Array (Agilent platform). (**E**) Taqman qRT–PCR analysis validating miR expression array results confirming upregulation of both miR-720 and miR-574-3p in the iASPP knockdown cells. The efficiency of the specific antagomirs was also measured. As a negative control, the qRT–pCR with miR-193a-3p shows no induction by sh-iASPP. Analysis of miR expression was carried out using the cycle threshold (Ct) method with RNU48 as endogenous control. The quantification of the miRNA samples is evaluated with the ΔΔCt method. $2^{(-\Delta\Delta Ct)}$ value is reported in the *y* coordinate in a linear scale. Data shown are mean values ± s.e.m. of each sample run in triplicate. (**F**) Western blot analysis showing that antisense miRNA (anti-miR) in HaCaT cells restores expression of p63. No effect on ΔNp63 expression was observed as a result of anti-miR-720 and miR-574-3p in sh-ctrl cells while a negative control anti-miR-193a-3p fail to reinduce expression of ΔNp63. In contrast, both antagomirs restored expression of p63 in cells knocked down for iASPP. (**G**) Expression of human p63 3′UTR in a luciferase reporter gene (pGL3 luc) leads to diminished luciferase activity in the presence of miR-720 and miR-574-3p, respectively, in HEK293 cells. (*$P<0.005$, *P*-values were obtained by using an one-sided Student's *t*-test). (**H**) qRT–PCR performed in primary keratinocytes treated with calcium to induce differentiation and involucrin, an epithelial differentiation marker was quantified. In the same experimental conditions, the quantifications of miR-574-3p and miR-720 were performed in the same samples. (**I**) Western blot analysis showing how transduction of anti-miR-720 or anti-miR-574-3p can prevents decrease of ΔNp63 during keratinocytes differentiation (mimic in culture with high serum calcium).

signalling (Carroll *et al*, 2006). Screening of various integrins using western blot analysis demonstrated significant modulation of these proteins by iASPP silencing, in particular β1 integrin (Figure 4A). These data support a role for iASPP in cell–matrix adhesion. Furthermore, p63 is an essential regulator of PERP, a critical component of the desmosome (Ihrie *et al*, 2005) and to support this we demonstrate that iASPP silencing significantly reduced PERP protein expression in keratinocytes (Figure 4B). Thus, we evaluated the effect of iASPP depletion on desmosomal proteins by western blot analysis and found significant dysregulation of desmosomal complexes (Figure 4B) which was confirmed at the mRNA level (Supplementary Figure S3B). Other adhesion complexes were also investigated and, for example, Claudin 1 (tight junction component) protein and Connexin 43 (gap junction component) protein confirmed the microarray

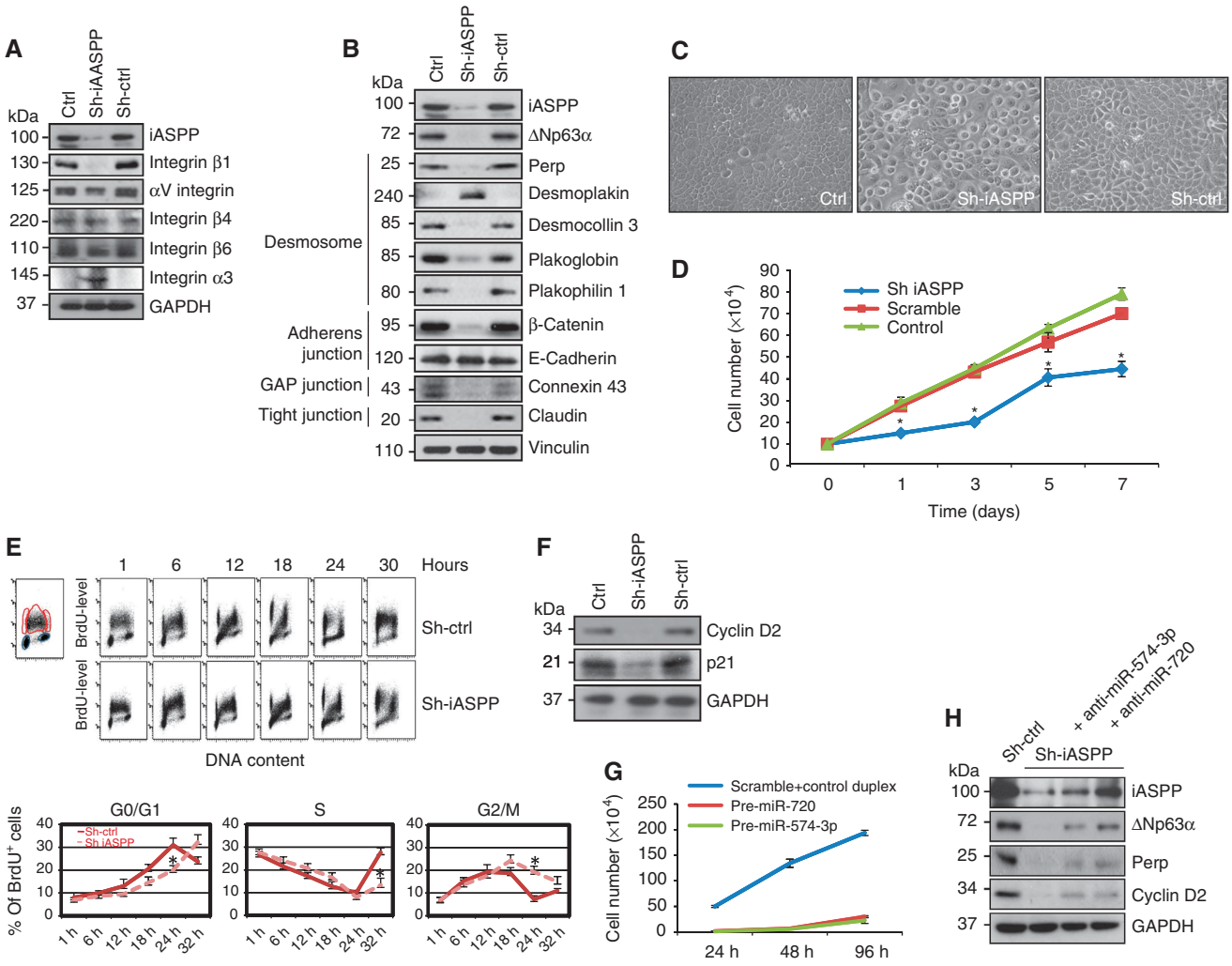

Figure 4 iASPP regulates genes related to epidermal adhesion and proliferation. (**A**) Western blot analysis of the integrin-associated proteins of CD47 showing the downregulation of β1 integrin, αV integrin, an upregulation of α3 integrin in the cells sh-iASPP compared with the controls while β4 and β6 integrins are unaffected. GAPDH is shown as a loading control. (**B**) Western blot analysis of cell adhesion proteins affected by iASPP knockdown. In the desmosomal proteins, we observed a downregulation of Perp, desmocollin 3, Plakoglobin and Plakophilin proteins in the cells depleted for iASPP compared with the controls while desmoplakin is upregulated. In the adherens junction, β-catenin protein is downregulated and E-cadherin is unaffected. Connexin 43 from the GAP junction and Claudin 1 from the tight junction are also downregulated. Vinculin is shown as a loading control. (**C**) Morphology of (i) control primary keratinocytes, transfected with (ii) siRNA-iASPP and (iii) siRNA-scramble using phase contrast microscopy (objective ×40). (**D**) Growth curves of cultured cells for sh-Scramble, sh-iASPP and control showing slower growth rates for shRNA-iASPP cells. (*$P<0.02$, *P*-values were obtained by using an one-sided Student's *t*-test). (**E**) Effect of iASPP depletion on cell cycle. Flow cytometric analysis of BrdU kinetics in shRNA-scramble versus shRNA-iASPP keratinocytes. Upper panel shows scatter plots for BrdU/DNA analysis over time from 1 to 30 h. Lower panel shows percentage of BrdU + ve cells (mean ± s.e.m.) in G0/G1, S and G2/M phases of the cell cycle. From 12 h, the G0/G1 fraction of $BrdU^+$ cells significantly decreased as a reflection of delayed progression of cells from S phase to G2/M and also delayed exit from the G2/M into a new cycle. At the last time point (30 h) compared with the control cells the accrued delay is about 6 h. Depletion of iASPP in this asynchronous keratinocyte cell line affects all phases of cell-cycle progression. (**F**) Downregulation of Cyclin D2 (validation from the array analysis) and p21 protein expression by western blot analysis performed in HaCaT sh-iASPP cells. GAPDH is shown as a loading control. (**G**) Growth curves of cultured primary keratinocytes transfected with control duplex, pre-miR-720 and pre-miR-574-3p showing how these specific miRNAs affect and reduce cell proliferation of the cells. (**H**) Western blot analysis showing how expression of specific anti-miR-574-3p and anti-miR-720 partially restore the protein levels of iASPP, ΔNp63, Perp and Cyclin D2 in iASPP-silenced cells; GAPDH is shown as a loading control.

analysis results. Overall, these data provide evidence that iASPP is crucial for maintaining the integrity of the same cell junction types in the epidermis as p63, thus strengthening our model of an autoregulatory feedback loop.

Morphological examination of keratinocytes was undertaken to assess the effect of sh-iASPP on cell growth (Figure 4C). Phase contrast microscopy showed that cells in which *PPP1R13L* is silenced demonstrate a strikingly altered morphology when compared with control keratinocytes, with loss of the typical 'cobblestone' morphology while acquiring the typical calcium-induced differentiation phenotype. iASPP depletion also led to a reduction in cell proliferation (Figure 4D) due to delayed cell-cycle progression. A bromodeoxyuridine (BrdU) kinetic analysis performed on asynchronous keratinocytes showed that depletion of iASPP altered cell-cycle progression; the G2/M fraction of BrdU + cells accumulated and delayed reentry to a new G0/G1 phase (Figure 4E). Furthermore, downregulation of *CCND2* mRNA and its protein product cyclin D2 confirmed microarray analysis data while cyclin-dependent kinase

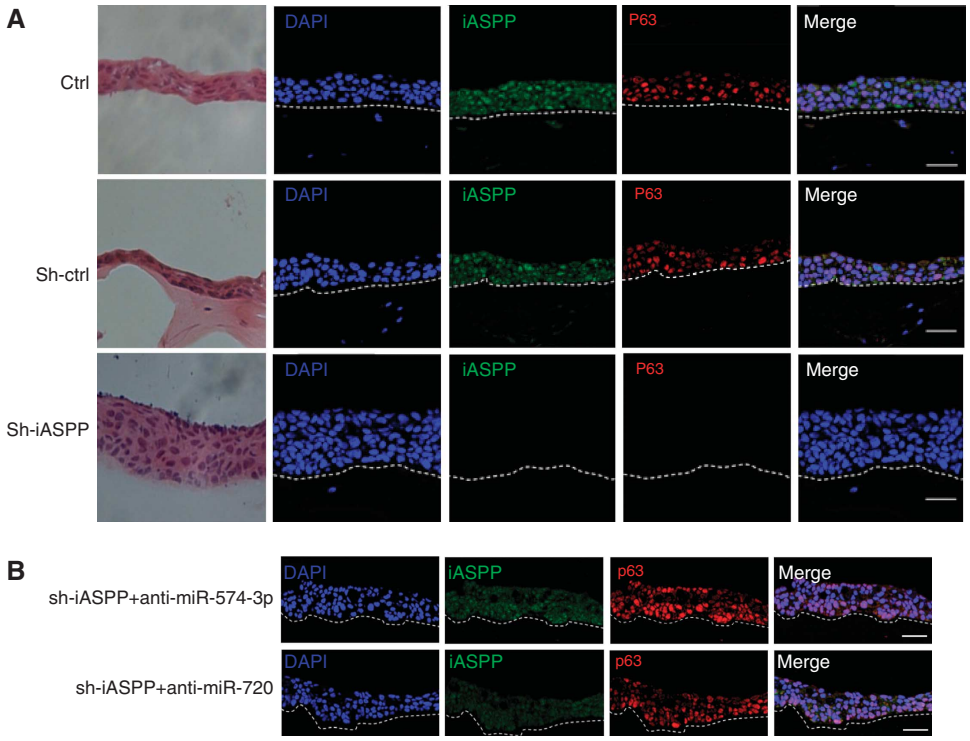

Figure 5 iASPP knockdown induces a multistratified epidermis. (**A**) H&E staining of 3D organotypic culture shows a thicker epidermis in the iASPP section compared with the control and Sh(RNA) scramble. Immunofluorescence staining confirms that silencing of iASPP results in the loss of p63 in the iASPP knockdown skin grafts section. DAPI (blue) is used as a nuclear marker. The dotted line represents the dermal–epidermal junction. Scale bar: 100 μm. (**B**) Immunofluorescence staining performed on organotypic 3D cultures where sh-iASPP keratinocytes were transduced prior to stratification with, respectively, anti-miR-574-3p and anti-miR-720. Their expression completely restores p63 protein levels and as a consequence iASPP one as well. DAPI (blue) is used as a nuclear marker. The dotted line represents the dermal–epidermal junction. Scale bar: 50 μm.

inhibitor, $p21^{Waf/Cip1}$ protein expression was also reduced by iASPP silencing (Figure 4F) confirming once again the independence of p53 in this autoregulatory feedback loop but also hinting to an activation of terminal differentiation process rather than having an effect on the cell cycle. Upregulation of pre-miR-720 or pre-miR-574-3p in primary keratinocytes is sufficient to significantly slow cell proliferation (Figure 4G). In order to validate the effect of the miRNAs previously described in the biological process affected by iASPP, silencing transduction experiments with specific antagomirs for miR-574-3p and miR-720 in keratinocytes partially restored endogenous protein levels of ΔNp63 and its target PERP as well as one of the cyclin D2 in sh-iASPP cells (Figure 4H). In addition, overexpression of iASPP in primary keratinocytes not only inhibits ΔNp63 degradation in the presence of high calcium conditions (differentiation condition) but also prevents increased expression of differentiation marker such as K1 and upregulates the basal cell marker K14 (Supplementary Figure S3C). These data not only reinforce the importance of iASPP/p63 axis in regulating the proliferative compartment but also show how iASPP expression could modulate the fine-tuning of epidermal homeostatic processes.

iASPP is involved in epidermal differentiation and stratification

These observations were further explored in 3D organotypic cultures, which represent an experimental model of skin reconstitution using keratinocytes cultured at the air–liquid interface on a dermal substrate. Examination of morphology of the stratified epithelia demonstrated that keratinocytes in which *PPP1R13L* was silenced by shRNA generated a markedly thicker epidermis when compared with controls (Figure 5A). Immunofluorescence staining also confirmed that in skin constructs depletion of iASPP correlates with loss of p63 expression (Figures 2F and 3A). Likewise transduction with specific antagomirs for miR-574-3p and miR-720, respectively, rescue p63 expression and as a consequence restore iASPP protein level in *PPP1R13L*-silenced skin reconstructions (Figure 5B).

To establish the effect of iASPP depletion in specific compartments of the epidermis, confocal immunofluorescence microscopy was performed using epithelial markers for adhesion, proliferation and differentiation. In control cells, strong reactivity of K14 and β1 integrin proteins was observed in the basal and suprabasal layers of the epidermis with positive Ki67 labelling of proliferating keratinocytes (Figure 6). The stratified epithelia were strongly positive for K1 whose expression corresponds to the spinous layer and confirmed by involucrin and loricrin expression, both markers of keratinocyte terminal differentiation, normally expressed in the suprabasal layers of stratified squamous epithelium. In contrast, in stratified skin constructs composed of iASPP-depleted keratinocytes, a premature differentiation process was observed. Immunostaining for K1 and involucrin and loricrin demonstrated that differentiation of sh-iASPP keratinocytes within the basal layer of the skin associated with concomitant loss of the proliferative

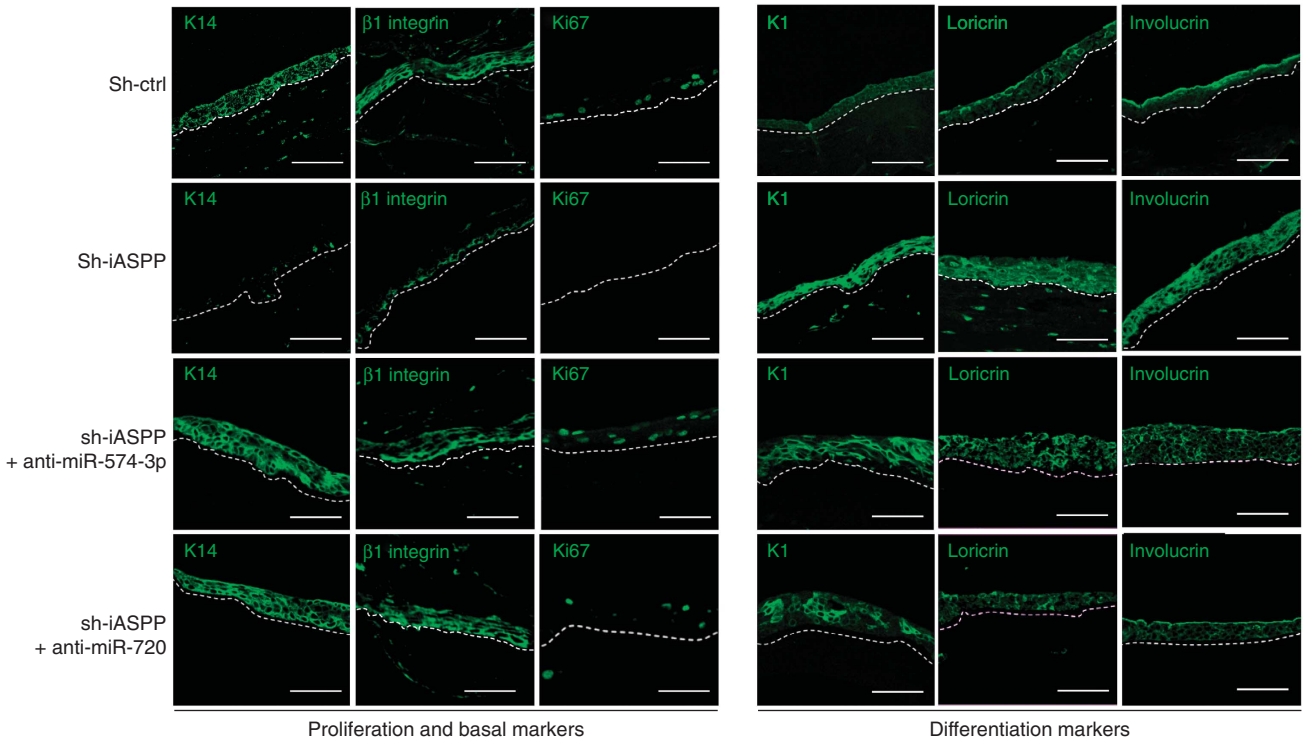

Figure 6 iASPP knockdown induces terminal differentiation. In sh-scramble sections, markers of differentiation, K1, loricrin and involucrin are detectable and display the normal basal K14, β1 integrin, and proliferative markers such as Ki67. In contrast, in sections with keratinocytes silenced for iASPP, the markers of differentiation are induced in all of the epidermis while basal and proliferation markers, were downregulated, highlighting the disorganization of the basal layer and occupancy by terminally differentiating cells. However, the sh-iASPP cells expressing the anti-miR-574-3p and anti-miR-720 show a reconstitution of the proliferative compartment with a positive staining of K14, β1 integrin, Ki67 and a reduction in the expression of the differentiation markers K1, loricrin and involucrin. The dotted line represents the dermal–epidermal junction. Scale bar: 50 μm.

compartment confirmed by decreased expression or absence of β1 integrin, K14 and Ki67 proteins.

The effect of iASPP on the proliferative compartment has been also assessed by comparing self-renewal potential of sh-iASPP keratinocytes respect their control in their ability to form colonies in monolayer culture. Cells depleted for iASPP display a reduction in the number of colony formation (Supplementary Figure S3D). Examination of size and colony morphologies classified in large clones, medium clones and small clones (Barrandon and Green, 1987) also results in a markedly reduction of large clones in iASPP-silenced cells (Supplementary Table S2) strengthening the importance of iASPP expression for the proliferative potential of epidermal cells.

Analysis of the iASPP-silenced skin reconstructs also showed that induction of specific antagomirs for miR-574-3p and miR-720 completely restore the expression patterns of the proliferation markers while partially rescuing the expression patterns of K1, involucrin and loricrin, due to the strong wave of terminal differentiation program activated by iASPP knockdown. Additionally, markers of cell adhesion integrity such as some of the desmosomal proteins were analysed in iASPP-silenced skin reconstructs confirming not only the previous monolayer data but also that specific induction of the antagomirs also partially restores the control expression patterns (Supplementary Figure S4). These results demonstrate that depletion of iASPP promotes and accelerates differentiation while simultaneously inhibiting the proliferation process in the stratified epidermal compartment of the skin, thereby explaining the thicker epidermis generated. Moreover, the data further confirm how the iASPP/p63 autoregulatory feedback loop is affecting these physiological processes in the skin via miR-574-3p and miR-720. *In situ* hybridization experiments were performed in order to localize the physiological expression of miRNA-574-3p and miRNA-720 in normal human epidermis. The staining patterns showed cytoplasmic and suprabasal expression for both miRNAs, confirming their link with the differentiation process (Figure 7A). A significant overexpression of both miRNA-574-3p and miRNA-720 is evident in stratified skin constructs composed of iASPP-depleted keratinocytes where their location and expression correlate with the stratification pattern, especially when compared with the poorly stratified controls (Figure 7B).

Discussion

iASPP is the most phylogenetically conserved inhibitor of p53 identified and its conservation through evolution emphasizes its important function (Bergamaschi *et al*, 2003). Our report provides new insights into the functional role of iASPP in human skin. This work began from examination of the developing epidermis in mouse embryogenesis. We found that iASPP was present from early stages of development to adult epidermis and partially colocalized in the nucleus with p63, the 'master initiator' of epithelial stratification (Koster *et al*, 2004). Moreover, we identified a novel autoregulatory feedback loop mechanism functioning between iASPP and

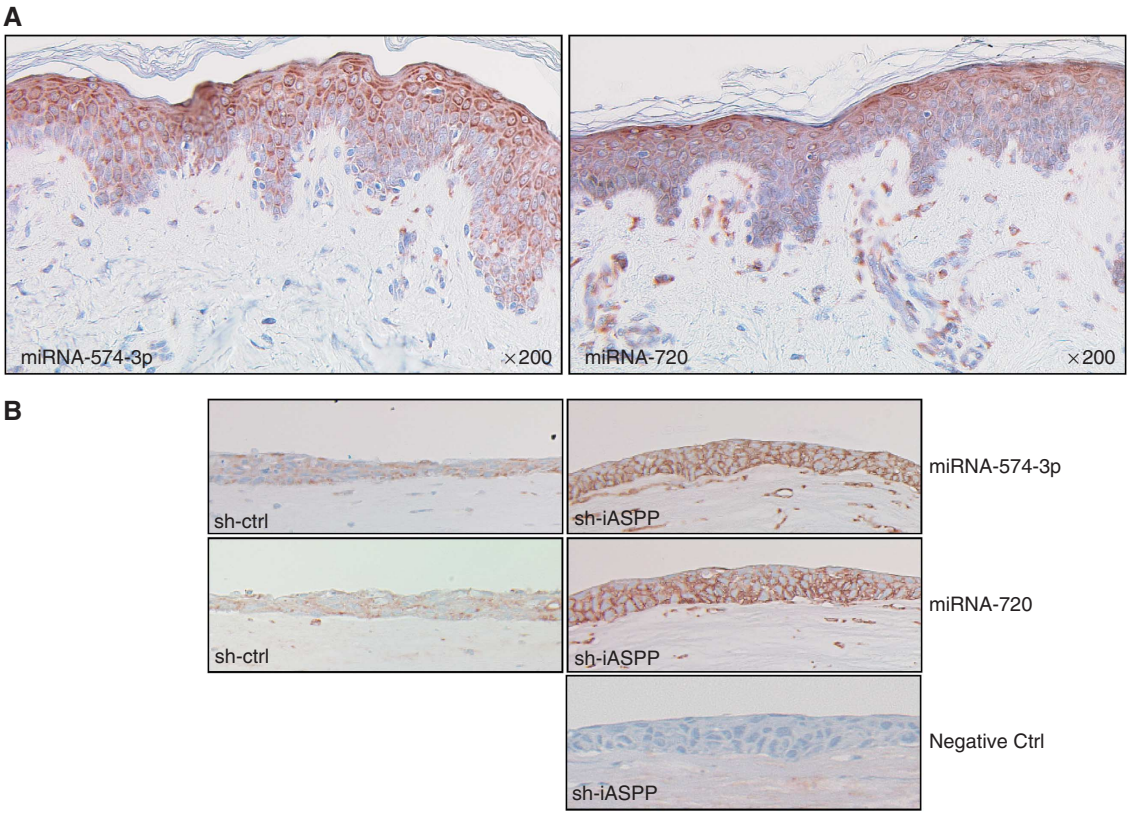

Figure 7 miR-574-3p and miR-720 are expressed in suprabasal layers in human epidermis. (**A**) *In situ* hybridization staining reveals clear cytoplasmic expression of miR-574-3p and miR-720 to suprabasal and differentiating layers of normal human skin. (**B**) *In situ* hybridization staining performed on the sections of skin grafts with sh-iASPP shows that silencing of iASPP allows relocation of both miR-574-3p and miR-720 everywhere in the epidermis. Phase contrast microscopy (objective × 200).

both TA and ΔNp63 in keratinocytes in which p63 modulates iASPP, and iASPP directly represses two unreported miRNAs, miR-720 and miR-574-3p which, in turn, control p63 protein expression by preventing its translation. Our model is summarized in Figure 8 and the physiological location of these two miRNAs in human epidermis indicates that they are linked to the activation of epidermal differentiation which correlates with recent work (Yi *et al*, 2008) identifying a skin miRNA that promotes differentiation by 'repressing stemness'. These data reveal iASPP as a crucial regulator of p63 in the skin independently of p53.

Our findings also identify novel biological roles in skin development and tissue homeostasis for iASPP, which acts via interplay with p63 to regulate genes essential for cell adhesion, differentiation and proliferation. p63 is a critical regulator of epidermal physiology, acting by modulating expression of cell adhesion-associated genes (Carroll *et al*, 2006) and regulating stem cell maintenance and differentiation of stratified epithelia (Truong *et al*, 2006; Senoo *et al*, 2007). Loss of p63 results in a complete absence of proliferating cells in the epidermis. Our data demonstrate that iASPP depletion targets genes such as β1 integrin, which is crucial for maintaining keratinocytes in the stem cell compartment and mediating their adhesiveness to the extracellular matrix (DiPersio *et al*, 1997; Kaur and Li, 2000; Grose *et al*, 2002). In addition, iASPP shares common targets of p63 such as Claudin 1 and Perp, junctional components essential for the epidermal barrier formation and cell–cell adhesion, respectively. We also showed that depletion of iASPP expression in keratinocytes results in a reduced proliferation of cultured epithelial cells. Taken together, our data suggest that the iASPP/p63 autoregulatory feedback loop is directly involved in controlling stem cell maintenance and also affects cellular junction components in keratinocytes.

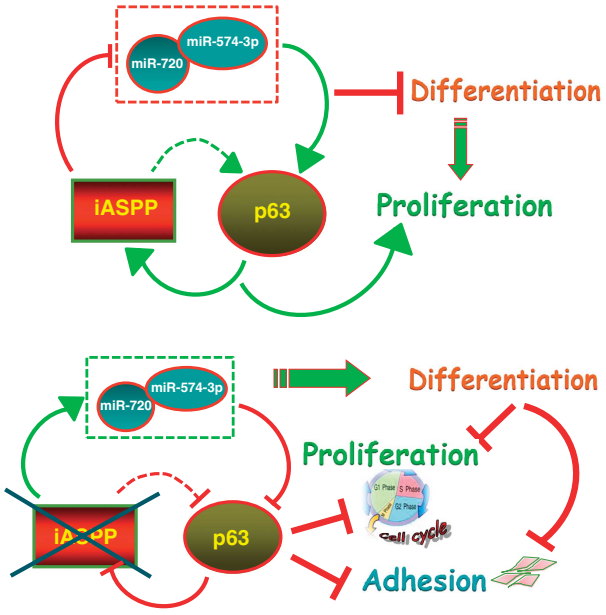

Figure 8 Proposed mechanism. Diagram illustrating how crucial physiological activities are affected by the iASPP/p63 autoregulatory feedback loop in keratinocytes.

iASPP depletion concomitantly reduced cyclin D2 expression in keratinocytes, which delays entry and progression of the cell cycle, but also cyclin-dependent kinase inhibitor p21 probably inducing terminal differentiation in a manner unrelated to its effects on the cell cycle (Di Cunto *et al*, 1998). A recent report highlighted that inactivation of *Trp63* in mice leads to increased expression of classical cell-cycle inhibitors such as p16INK4A and p19ARF (Su *et al*, 2009). These data provide evidence of a role for iASPP in regulating the cell cycle in keratinocytes through p63.

When maintained in monolayer, keratinocytes silenced for iASPP are flattened and adherent resembling the calcium-induced differentiation phenotype. Since proteins involved in cell adhesion are modulated during the epidermal stratification process, we assessed expression levels of cell adhesion components. A recent study analysed and classified genes affected by calcium treatment in keratinocytes using the SOURCE database of Stanford genes (Seo *et al*, 2005). In the category 'cytoskeleton and cell adhesion', Desmoplakin was upregulated while β1 integrin was among the genes downregulated. Our data is in line with these results, suggesting that keratinocytes depleted for iASPP display some of the features of terminal differentiation.

The organotypic skin model allowed reconstitution of the epidermis to visualize putative *in vivo* effects of iASPP silencing, which resulted in failure of maintenance of the proliferative compartment controlled by key genes such as *p63* and also misassembly of the elaborate architecture governed by junctional proteins such as desmocollin 3 and β-catenin (Hardman *et al*, 2005). Interestingly, iASPP depletion results in an abnormal differentiation program in cells within the basal layer by promoting and accelerating stratification. From the monolayer data showing how iASPP could affect the integrity of the junctional complex, a more severe epidermal phenotype might have been predicted in organotypic cultures. Instead, the data clearly showed a thicker epidermis, which reflects how iASPP silencing in keratinocytes converts them to differentiated cells. The proliferative compartment of the basal layer is lost and keratinocytes then behave like spinous and granular cells, mainly becoming committed to epidermal stratification. These data suggest that silencing of iASPP disrupts the maintenance of the stem cells in the basal compartment of the epidermis, increasing the proportion of the transient amplifying cell population. These cells move upwards to the spinous and granular layers and committed to differentiation (Yang and McKeon, 2000; Waikel *et al*, 2001; Frye *et al*, 2003). In support of this observation, a model in which p63 function maintains the regenerative capacity of epithelial stem cells has been reported (Senoo *et al*, 2007). Indeed, $p63^{-/-}$ cells undergo differentiation by a massive depletion of stem cells.

Mutations targeting proteins of cell junction integrity are responsible for human syndromes characterized by cardiomyopathy, woolly hair and palmoplantar keratoderma. The most well-studied examples of this phenotypic triad, the autosomal recessive conditions Naxos disease and Carvajal syndrome, are caused by mutations in desmosomal structural genes such as plakoglobin (McKoy *et al*, 2000) and desmoplakin (Norgett *et al*, 2000), respectively. Naxos-like syndromes have also been associated with other desmoplakin mutations, both recessive (Alcalai *et al*, 2003; Uzumcu *et al*, 2006) and dominant (Norgett *et al*, 2006). In some cases, mutations in the genes encoding plakoglobin (*JUP*), desmoplakin (*DSP*) or other desmosomal genes have not been detected (Djabali *et al*, 2002; Alonso-Orgaz *et al*, 2007), suggesting that mutations in *PPP1R13L* in mice and cattle cause phenotypically similar cardiocutaneous disease and both mutations were predicted to have a severe impact on cellular function of the iASPP protein product (Herron *et al*, 2005; Simpson *et al*, 2009). Remarkably, iASPP depletion also targets *PERP*, a p63 target gene and critical component of the desmosome, required for the integrity of the stratified epithelia (Ihrie *et al*, 2005). These data support the importance of iASPP in regulating genes involved in the junctional complex in the skin and also highlight the need to investigate mutations in iASPP in human cardiocutaneous syndromes and non-syndromic forms of cardiomyopathy in which mutations of desmosomal genes are not detected.

The phenotype we observe following depletion of iASPP raises some discrepancies with previous reports. p63 loss has been associated with cell detachment/anoikis linked with the loss or decrease of β4 integrin expression, while ectopic expression of β4 integrin significantly reduced apoptosis following p63 knockdown (Carroll *et al*, 2006). In another report, α3 integrin was suppressed in human primary keratinocytes by both p63 knockdown and differentiation (Kurata *et al*, 2004). In contrast, in our system β4 integrin expression was unaffected by iASPP silencing (and consequent p63 loss), whereas α3 integrin expression was upregulated. Reflecting the differences in gene expression, our model also differs from published data indicating that depletion of all p63 isoforms in organotypic cultures results in hypoproliferation and loss of differentiation (Truong *et al*, 2006). Instead, p63 loss resulting from iASPP depletion prevents proliferation and surprisingly activates a premature differentiation stream. We cannot exclude that iASPP is acting upstream of p63 and as such modulates its downstream activities, but our data suggest that iASPP also may be involved in alternative signalling pathways leading to a more complicated phenotype. Further studies will aim to elucidate the complex molecular pathways involved in the development of the epidermal phenotype following depletion of iASPP.

iASPP is certainly crucial for the fine-tuning of homeostasis in the basal layer of epidermis and this raises the question whether iASPP might be involved in the aging process by influencing differentiation and proliferation, together with the integrity of the cell junctional components. Further investigation in disease could provide a better understanding of the molecular pathways involved in this process. Nonetheless, iASPP expression is frequently increased in cancer; our findings might provide a molecular mechanism for tumourigenesis, and thus have implications for the future design of therapeutic approaches.

Materials and methods

Cell culture, transfection and DNA damaging treatments

Primary keratinocytes were isolated from neonatal foreskin, cultured in keratinocyte serum-free medium (Gibco) with 2 mg/ml Glutamine, 0.3 mM calcium chloride, 2.5 mg human recombinant EGF and 25 mg bovine pituitary extract. HEK293, Phoenix and HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Neb-1 and N-TERT

cells were cultured in DMEM: F12 media supplemented with FBS 20%, 1% glutamine, 50 μg/ml gentamicin, 100 μM TPA, 2 μg/ml hSCF, 0.1 μM cholera toxin and 10 μM endothelin. HEK293 cells were transfected with Lipofectamine 2000 reagent (Invitrogen) at a 1:2 (μl/μg) ratio with DNA using 5 μg of plasmid DNA. Down-regulation of iASPP was obtained by using pSuper retro-puro vector (OligoEngine) to express stable shRNA. Retroviral stocks were generated from Phoenix cells transfected with iASPP constructs using Fugene 6 as described by the manufacturer. Cells positively transfected were selected with Puromycin (Sigma) until reaching 100% of confluence and virus-containing medium was harvested 18 h after induction at 32°C, filtered, aliquoted and stored at −80°C. N-TERT cells and HaCat cells were infected with shRNA viruses for each condition (scrambled control and three iASPP-specific shRNAs). Selection and growth were done with Puromycin treatment. Cells were treated with UV-B as indicated in figure legends. MG132 (Sigma) was used as indicated in figure legends.

Antibodies

The antibodies used were KDEL ER marker mouse monoclonal, Golgi mouse monoclonal, Proteosome 20S rabbit polyclonal, GAPDH rabbit monoclonal, β1 integrin, β4 integrin, αV integrin, β6 integrin and α3 integrin from Abcam; anti-p63 4A4 from Neomarkers (for WB) and H129/H137 from Santa Cruz (for ChIP and immunofluorescence); anti-TA-p63 kindly provided by Dr Eleonora Candi; anti-E-Cadherin, anti-PERP, anti-Vinculin and anti-iASPP clone LXO49.3 from Sigma; K1, K10, Involucrin and Ki67 antibodies (gifts from Professor Irene Leigh); β-catenin from Cell Signaling; anti-Desmoplakin (11-5F) generous gift from Prof D Kelsell; anti-desmocollin 3 (U114) and Plakophilin-1(PP1-5C2) from Progen; Plakoglobin (PG 5.1) from Serotec.

Cell proliferation assay

Sh-iASPP cells, Sh-Scrambled (Sh-ctrl) cells and control cells (2×10^4) were seeded onto six-well plates in DMEM containing 10% FBS. Proliferation was assessed at different days.

Organotypic cultures

Organotypic cultures on collagen: matrigel gels were performed as previously described with some modifications (Nyström *et al*, 2005). Collagen: matrigel gels were prepared by mixing 3.5 volumes of type I collagen (First Link, UK), 3.5 volumes of Matrigel (BD Biosciences, UK), and 1 volume of 10 × DMEM, 1 volume of FCS and 1 volume of DMEM with 10% FCS/HFF (resuspended at a density of 5×10^6/ml). One microlitre of the gel mixture was placed into each well of a 24-well plate and allowed to polymerase at 37°C for 1 h. After polymerization, 1 ml of DMEM was added per well and gels were incubated for 18 h to equilibrate. Keratinocytes with iASPP shRNA, scramble shRNA and control were seeded into a plastic ring placed on the top of the gel at a density of 5×10^5 cells/gel. After 24 h, the rings were removed and gels were raised to the air–liquid interface on stainless steel grids. The gels were harvested at day 14 and frozen at −80°C.

Luciferase assay

HEK293 cells were seeded in triplicate at a density of 2×10^5 in a 12-well plate. The following day, cells were transfected with 1 μl of Lipofectamine 2000 per well in serum-free medium. The following plasmids were used: pGL3-Control and pGL3-containing a p63 3′UTR-firefly luciferase reporter. Each plasmid was co-transfected with 80 nM of each of the following microRNAs: miR-720 and miR-574-3p (Ambion). Six hours after transfection, serum-free medium was replaced by serum-containing medium. Cells were lysed 48 h after transfection by using the manufacturer's protocol for the Dual Luciferase Assay (Promega). The experiment was repeated three times. Efficiency of transfection was normalized using Renilla luciferase activity.

RNA interference

For iASPP knockdown, primary keratinocytes, HaCaT, N-TERT and Neb1 cell lines were transfected with a SMARTpool of four siRNAs (Dharmacon, UK), targeting iASPP (L-003815-00-0020). For p63 knockdown, siRNA sequences p63 siRNA (ID 217143, ID 4893 and ID 217144; Applied Biosystems) were used in combination for siRNA-p63 pool while for a specific sequence for siRNA-TAp63 was kindly provided by Gerry Melino's laboratory. Transfection was performed according to the manufacturer's protocol and optimized for a six-well plate. Briefly, cells were plated at 50% confluency subjected to transfection on the following day using 3.25 μl of DharmaFECT1 (Dharmacon, UK) transfection reagent and 60 nM final concentration of each siRNA. Transfection media were replaced with complete DMEM media after 24 h. iASPP or p63 protein expression was analysed by WB after 48 h post-transfection. Cells incubated with the transfection reagent only (control) as well as cells transfected with a pool of non-targeting siRNAs (si*CONTRO*L Non-Targeting siRNA Pool) were used as negative controls.

Microarray analysis

GeneChip Human Genome U133A 2.0 Arrays (Affymetrix) and Whole Human Genome 4 × 44K Oligo Microarrays (Agilent Technologies) were used to compare the expression profiles of keratinocytes with or without iASPP silencing. Agilent protocol: for each sample and reference pair, equal amounts of mRNA were amplified by means of the Amino Allyl MessageAmp II aRNA Kit (Ambion Inc., Austin, TX) to obtain amino allyl antisense RNA following the method developed by Eberwine and co-workers (Van Gelder *et al*, 1990). One round of amplification was performed and both dsDNA and aaRNA underwent a purification step using columns provided with the kit. Labelling was performed using NHS ester Cy3 or Cy5 dyes (GE HealthCare, Buckinghamshire, UK) able to react with the modified RNA. Hybridization with dye-swap duplication was performed to compare sample versus reference. The same quantity of differentially labelled sample and reference was put together, fragmented and hybridized to oligonucleotide glass arrays with sequences representing >41K human unique genes and transcripts. All steps were performed using the Gene Expression Hybridization kit (Agilent Technologies) following the 60-mer oligo microarray processing protocol (Agilent Technologies). Slides were washed with the Agilent wash procedure and scanned with the dual-laser microarray scanner Agilent G2505B (Agilent Technologies). Images were then analysed using Feature Extraction software (Agilent Technologies) version 9.5. TotRNA and mRNA quality, concentration and labelling were checked by means of RNA 6000 nano chip assays (Agilent Technologies) and Agilent 2100 Bioanalyzer and NanoDrop ND-1000 Spectrophotometer (Thermo-Scientific, Wilmington, DE, USA). Affymetrix .CEL files and Agilent Feature Extraction output files were loaded into the Resolver SE System (Rosetta Biosoftware, Seattle, WA). Data processing and normalization were performed using platform-specific error models.

miRNA analysis

Samples were labelled and hybridized according to Agilent Technologies miRNA Microarray System protocol. Briefly, 100 ng of total RNA was dephosphorylated with Calf Intestinal Phosphatase at 30°C for 30 min. The reaction was stopped adding DMSO, incubating at 100°C for 10 min and immediately transferred on an ice bath. Samples were labelled with a ligation step, using T4 RNA ligase and Cyanine3-pCp and incubating at 16°C for 2 h. After the reaction, the samples were completely dried in a vacuum concentrator. For hybridization, samples were resuspended in nuclease-free water. After adding the provided Blocking Agent and Hybridization Buffer, reactions were incubated at 100°C for 5 min and immediately transferred on an ice bath for 5 min. Hybridization on miRNA glass arrays (Human miRNA Microarray Release 12.0, Agilent Technologies) was carried out for 20 h in a hybridization oven set at 55°C, with a rotation speed of 20 r.p.m. After incubation, slides were washed following Agilent protocol and immediately scanned with the Dual-Laser Microarray Scanner B (Agilent Technologies). Images were then analysed using Feature Extraction software (Agilent Technologies) version 9.5. A linear intensity-dependent normalization was performed on combined intensity values of treated and control samples, and miRNAs with log-fold change >1 were selected for further analyses.

ChIP assay

ChIP experiments were performed in keratinocytes, crosslinked for 15 min with 1% formaldehyde, neutralized with 125 mM glycine pH 2.5 and washed in PBS. After preparation and lysis of nuclei, with chromatin sonication to 500–1000 base pairs (bp) average fragment size, 1 mg of protein extract was preclear with protein-agarose/salmon sperm (Millipore) for 1 h with rocking at 4°C, then immunoprecipitated with p63 antibodies overnight. The day after, immunocomplexes were washed and proteins were reverse

crosslinked overnight at 56°C in 2.5 M NaCl and RNase A at 10 μg/μl, then proteins were degraded in 0.5 M EDTA, 20 μl 1 M Tris pH 6.5 and 2 μl Proteinase K 10 mg/ml 1 h at 45°C. DNA extraction was performed with phenol–chloroform. A negative control for immunoprecipitation was performed in the presence of isotype-specific unrelated antibody. Purified DNA was resuspended in water and subjected to PCR. Primers sequence used: p53 BS F: 5′-GGAGTGGAGAGGACTCAACG-3′, p53 BS R 5′-CTGCTGCATCGAC CACTTAG-3′; TK F: 5′-GTGAACTTCCCGAGGCGCAA-3′, TK R: 5′-GC CCCTTTAAACTTGGTGGGC-3′; p21 F: 5′-ATGTATAGGAGCGAAGGT GCA-3′, p21 R: 5′-CCTCCTTTCTGTGCCTGAAACA-3′.

BrdU staining

Cells in culture were incubated with 10 μM BrdU (Sigma) for 30 min, washed with PBS and afterwards harvested at different times from the BrdU incorporation (1, 6, 12, 18, 24 and 30 h). Cells were then washed in PBS, trypsinized, pelleted and resuspended in ice-cold 70% ethanol for 30 min at 4°C. Fixed samples were then pelleted, resuspended in 2 M HCl at room temperature for 30 min. Cells were washed in PBS supplemented with 0.1% BSA and 0.2% Tween-20, pH 7.4, incubated with 2 μl anti-BrdU (Becton Dickinson) at room temperature for 20 min, then washed in PBS–Tween, incubated with secondary antibody conjugated with Alexa (Molecular Probes) for 20 min at room temperature. Samples were washed in PBS, incubated with 50 μl RNAse (100 μg/ml, Sigma) at 37°C for 15 min and then incubated with Propidium iodide (50 μg/ml, Sigma).

Colony forming assay

HaCaT cells depleted for iASPP and scrambled control were seeded in 6 cm plates at a cell density of 500 cells/well. After 10 days, cells were fixed, stained with crystal violet and scored for colony formation. Morphology and colony size analysis were also performed to further characterize the holo-mero-para clones.

In situ hybridization

The oligonucleotides for *in situ* hybridization of miR-574-3p and miR-720 were purchased as LNA probes from Exiqon. Labelling of the oligonucleotides was done with 'DIG Oligonucleotide 3′-End Labelling Kit' (Roche Diagnostics, Mannheim, Germany). The control oligonucleotide from the Roche Kit was used as negative control. The labelling reaction was carried out as described in the manufacturer's protocol. Deparaffinization of 4 μm thick tissue sections was done by incubation in xylene twice for 5 min. Samples were hydrated through a descending alcohol series (100, 90, 70%; 5 min each). Permeabilization of tissue was reached by treating the sections with 0.2% pepsin (Sigma-Aldrich) in 0.2 M HCl for 15 min at 37°C and stopped by incubating two times in 0.1 M Glycine (Roth, Karlsruhe, Germany) in PBS for 3 min at RT. After two washes with PBS, DNA was digested with 50 units/ml DNase (Fermentas) in the associated buffer for 1 h at 37°C, followed by a short wash step with PBS. Endogenous peroxidases were inactivated by incubation in 3% H_2O_2 in Methanol for 20 min. Afterwards, sections were washed twice with dest. H_2O and twice with 2 × SSC (0.3 M NaCl, 0.03 M Na-citrate), 3 min each. The prehybridization mix (50% Formamide, 2 × SSC, 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.5% SDS, 1 mM EDTA, 10 μg salmon sperm DNA) was heated for 2 min at 90°C and put on the slides, which were then put on a heat plate at 90°C for 2 min. After 1–2 h incubation at RT, the prehybridization mix was exchanged with 90°C preheated prehybridization mix with 1 pmol/50 μl digoxigenin-labelled probe. The slides were put on a heat plate at 90°C for 2 min and incubated overnight at RT. Next day slides were washed twice with 2 × SSC for 15 min at 37°C, once with 1 × SSC for 15 min at 37°C and once with 0.5 × SSC for 15 min at 37°C. Detection of digoxigenin-labelled probes was done with anti-digoxigenin-POD Fab fragments (Roche) in a 1:100 dilution and TSA Biotin System (Perkin-Elmer, Waltham, USA) according to the manufacturer's protocol. Afterwards, AEC staining was done on the slides for 1 min. Sections were counterstained with haematoxylin (Sigma-Aldrich) and embedded with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: AC designed and performed most of the experiments, with some contributions from VS, RNHM and DL. MH performed the *in situ* hybridization, while CR, AB and SK provided technical assistance. PO, MM-G, CG and GC analysed the microarray and miRNA assay. BA, GC, KMB, MPP and CAH provided intellectual support. DB planned the project, designed experiments and wrote with AC the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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