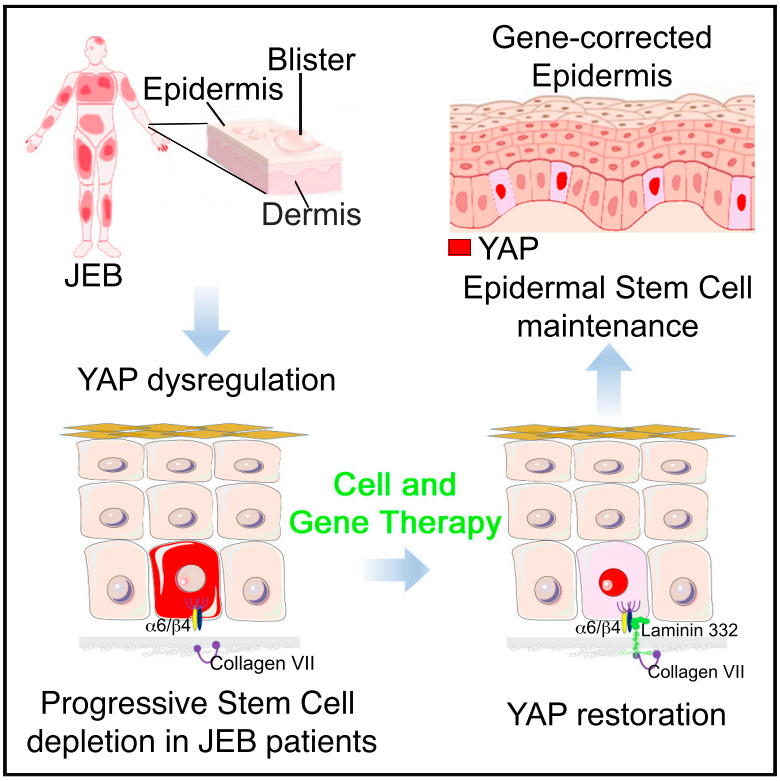
Article

Cell Reports

Laminin 332-Dependent YAP Dysregulation Depletes Epidermal Stem Cells in Junctional Epidermolysis Bullosa

Graphical Abstract



Authors

Laura De Rosa, Alessia Secone Seconetti, Giorgio De Santis, ..., Graziella Pellegrini, Johann W. Bauer, Michele De Luca

Correspondence

michele.deluca@unimore.it

In Brief

Gene therapy of junctional epidermolysis bullosa is hampered by the epidermal stem cell loss marking this disease. De Rosa et al. find that YAP dysregulation underpins such loss, which is recapitulated by laminin 332 and α6β4 integrin ablation. Combined cell and gene therapy rescues adhesion, YAP function, and stem cells.

Highlights

- YAP sustains epidermal stem cells
- *LAMB3*-dependent JEB leads to YAP inactivation and epidermal stem cell depletion
- JEB cell and gene therapy rescue adhesion, YAP, and stem cells *in vitro* and *in vivo*
- Enforced YAP rescues stem cells in the absence of cell adhesion

De Rosa et al., 2019, Cell Reports *27*, 2036–2049
May 14, 2019
https://doi.org/10.1016/j.celrep.2019.04.055

CellPress

OPEN ACCESS
CellPress

Cell Reports
Article

Laminin 332-Dependent YAP Dysregulation Depletes Epidermal Stem Cells in Junctional Epidermolysis Bullosa

Laura De Rosa,[1,7,8] Alessia Secone Seconetti,[1,7,8] Giorgio De Santis,[2] Giovanni Pellacani,[3] Tobias Hirsch,[4,9] Tobias Rothoeft,[5] Norbert Teig,[5] Graziella Pellegrini,[1,3] Johann W. Bauer,[6] and Michele De Luca[1,10,*]

[1]Centre for Regenerative Medicine "Stefano Ferrari," Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
[2]Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, Modena, Italy
[3]Department of Surgery, Medicine, Dentistry, and Morphological Sciences, University of Modena and Reggio Emilia, Modena, Italy
[4]Department of Plastic Surgery, Burn Centre, BG University Hospital Bergmannsheil, Ruhr-University Bochum, Germany
[5]Department of Neonatology and Pediatric Intensive Care, University Children's Hospital, Ruhr-University Bochum, Germany
[6]EB House Austria and Department of Dermatology, University Hospital of the Paracelsus Medical University, Salzburg, Austria
[7]These authors contributed equally
[8]Present address: Holostem Terapie Avanzate, s.r.l, Modena, Italy
[9]Present address: Department of Plastic, Reconstructive, and Aesthetic Surgery, Hand Surgery, Fachklinik Hornheide, and Division of Plastic Surgery, Westfaelische-Wilhelms-University of Muenster, Muenster, Germany
[10]Lead Contact
*Correspondence: michele.deluca@unimore.it
https://doi.org/10.1016/j.celrep.2019.04.055

SUMMARY

Laminin 332-deficient junctional epidermolysis bullosa (JEB) is a severe genetic skin disease. JEB is marked by epidermal stem cell depletion, the origin of which is unknown. We show that dysregulation of the YAP and TAZ pathway underpins such stem cell depletion. Laminin 332-mediated YAP activity sustains human epidermal stem cells, detected as holoclones. Ablation of YAP selectively depletes holoclones, while enforced YAP blocks conversion of stem cells into progenitors and indefinitely extends the keratinocyte lifespan. YAP is dramatically decreased in JEB keratinocytes, which contain only phosphorylated, inactive YAP. In normal keratinocytes, laminin 332 and α6β4 ablation abolish YAP activity and recapitulate the JEB phenotype. In JEB keratinocytes, laminin 332-gene therapy rescues YAP activity and epidermal stem cells *in vitro* and *in vivo*. In JEB cells, enforced YAP recapitulates laminin 332-gene therapy, thus uncoupling adhesion from proliferation in epidermal stem cells. This work has important clinical implication for *ex vivo* gene therapy of JEB.

INTRODUCTION

Constant regeneration and repair of the human epidermis rely on specific long-lived stem cells, which generate short-lived, transient-amplifying (TA) progenitors that undergo terminal differentiation (Hirsch et al., 2017). Interfollicular stem and TA cells reside in the epidermal basal layer and adhere to the basement membrane through hemidesmosomes, specialized junctional complexes linking the extracellular matrix to keratins (Walko et al., 2015). Within hemidesmosomes, α6β4 integrins bind to laminin 332 (also known as laminin 5), a heterotrimeric protein consisting of α3, β3, and γ2 chains. In turn, laminin 332 tightens the epidermal-dermal junction by binding to the NC-1 domain of type VII collagen, the major component of the dermal anchoring fibrils linking the basement membrane to anchoring plaques in the papillary dermis (Borradori et al., 1997; De Luca et al., 1990; Hopkinson and Jones, 2000; Rousselle et al., 1997; Sonnenberg et al., 1991; Stepp et al., 1990).

Epidermolysis bullosa (EB) is a group of devastating, often early lethal, genetic disorders characterized by structural and mechanical fragility of skin and mucosal membranes. Recurrent blistering and multiple infections, chronic skin wounds, and disabling scarring severely impair the quality of life of EB patients (see Fine et al., 2014, 2008 for review). The most severe forms of the disease are generalized junctional EB (JEB) and recessive dystrophic EB (RDEB). JEB is caused by mutations in *LAMA3*, *LAMB3*, or *LAMC2* (which, together, encode laminin 332); *ITGA6* and *ITGB4* (encoding α6β4 integrins); and *COL17A1* (encoding collagen XVII, also known as bullous pemphigoid antigen 2). RDEB is due to mutations in *COL7A1* (encoding collagen VII). Thus, the skin cleavage occurs within hemidesmosomes in JEB and within the superficial dermis in RDEB (Fine et al., 2014). There is no cure for EB, and symptomatic treatments aim at relieving the ravaging clinical manifestations.

Autologous cultures of human keratinocytes (Rheinwald and Green, 1975) containing a defined number of stem cells have long been used to prepare grafts that can permanently restore massive epithelial defects, such as skin and corneal burns (Gallico et al., 1984; Pellegrini et al., 1999b, 1997; Rama et al., 2010; Ronfard et al., 2000). *LAMB3*-deficient JEB (hereafter referred to as JEB) has been tackled through transplantation of transgenic epidermal cultures (Bauer et al., 2017; De Rosa et al., 2013; Mavilio et al., 2006). RDEB gene therapy has been

developed as well (Siprashvili et al., 2016). Combined *ex vivo* cell and gene therapy has proved to be lifesaving, as it was able to regenerate virtually an entire, fully functional epidermis on a child suffering from a devastating form of JEB with poor prognosis (Hirsch et al., 2017). Despite the successful outcome of these trials, we repeatedly observed that epidermal cells obtained from JEB patients initiate colonies with low efficiency. Once (and if) established, JEB cultures are often depleted of detectable stem cells and/or show very rapid clonal conversion leading to premature stem cell depletion and replicative senescence, making *ex vivo* gene therapy particularly cumbersome and sometimes not doable. Of note, these features were particularly evident in JEB adults and were not observed in cultures initiated from other forms of EB. This notion prompted us to investigate whether JEB stem cell depletion might be ascribed to a perturbation of signals emanating from the basal keratinocyte adhesive machinery.

The Yes-associated protein (YAP) and its paralog with PDZ-binding motif (TAZ) are transcriptional co-activators driving cell proliferation in many types of stem and progenitor cells and cancers and key regulators of mechanotransduction (see Harvey et al., 2013; Irvine and Harvey, 2015; Pan, 2010; and Piccolo et al., 2013 for review). Unphosphorylated YAP translocates to the nucleus, where it induces target genes through interaction with TEAD transcription factors. Phosphorylation of YAP and TAZ by Mst1/2 and Lats1/2 kinases in defined serine residues results in their sequestration, hence functional inactivation, into the cytoplasm by 14-3-3 proteins (Zhao et al., 2007). This molecular system, referred to as the Hippo signaling pathway, senses adhesion-dependent mechanical cues (see Dobrokhotov et al., 2018; Dupont, 2016 for review).

In this paper, we show that (1) adhesion-dependent, laminin 332-mediated YAP activity is instrumental in sustaining human epidermal stem cells; (2) JEB triggers YAP inactivation, leading to epidermal stem cell depletion; (3) gene therapy-mediated restoration of JEB adhesive properties rescues YAP activity and epidermal stem cells *in vitro* and *in vivo*; and (4) enforced YAP mimics *LAMB3*-gene therapy and decouples epidermal adhesion from stemness.

RESULTS

Nuclear YAP Is Selectively Expressed in Human Epidermal Stem Cells

An important step toward molecular definition of human epidermal stem cells came from the discovery of p63 (Yang et al., 1998) as a key transcription factor sustaining all squamous epithelia (Mills et al., 1999; Yang et al., 1999). In particular, ΔNp63α (hereafter referred to as p63α) underpins the proliferative, regenerative capacity of epithelial stem cells (Senoo et al., 2007; Yang et al., 1999). In normal human skin, nuclear (active) YAP was detected almost exclusively in p63α^{bright} basal keratinocytes (Figure 1A, at arrows). The basal layer contained a mean of 30.6% ± 6.7 p63α^{bright}/YAP^{nuclear} keratinocytes (average of 5 donors) (Figure 1A, inset). Basal p63α^{dim} and basal and suprabasal p63α^{neg} keratinocytes contained only cytoplasmic (inactive) YAP (Figure 1A, at asterisks).

Human keratinocytes initiate different types of colonies, referred to as holoclones, meroclones, and paraclones (Figure S1; see STAR Methods) (Barrandon and Green, 1987; Pellegrini et al., 1999a); p63α strongly declines during clonal transition from holoclone to meroclone, and the protein is virtually absent in paraclones (Di Iorio et al., 2005; Pellegrini et al., 2001). Clonal tracing of human transgenic epidermis unambiguously showed that holoclone-forming cells are self-renewing, long-lived stem cells underpinning the epidermis and giving rise to pools of short-lived progenitors (meroclones and paraclones) that ultimately replenish differentiated cells and contribute to wound healing (Hirsch et al., 2017). Strikingly, the long-term clinical success of epithelial cultures relies on a defined number of p63α^{bright} holoclones (Pellegrini et al., 2013; Rama et al., 2010). Holoclone-type colonies—defined as round colonies with smooth and regular borders and formed entirely by small cells with scarce cytoplasm—of a 4-day epidermal primary culture were formed mainly by small p63α^{bright} keratinocytes containing nuclear YAP (Figure 1B). At longer culture time (6–10 days), keratinocytes gather at the center of the colonies and start to stratify and differentiate. In such colonies, nuclear YAP was expressed by peripheral, proliferating p63α^{bright} cells (Figure 1B, at arrows), whereas central keratinocytes contained only cytoplasmic, inactive YAP and were either p63α^{dim} or p63α^{neg} (Figure 1B, at asterisks).

Of note, nuclear YAP was tightly related to the expression of 14-3-3σ and α-catenin, the latter being a component of adherens junctions sensing epidermal cell density and restricting keratinocyte proliferation (Schlegelmilch et al., 2011). Keratinocytes located at the edge of growing colonies contained nuclear YAP and virtually undetectable 14-3-3σ, while cells crowded at the center of the colony expressed 14-3-3σ and contained only cytoplasmic YAP (Figure 1C, 14-3-3σ/YAP, at asterisks). As shown in Figure 1C (4 days), small colonies, formed predominantly by YAP^{nuclear} cells, expressed low amounts of α-catenin located at cell-cell junctions (at arrow). Actin microfilaments-enriched cells, located at the center of growing colonies (6–12 days), expressed high amounts of α-catenin and contained only cytoplasmic YAP. Note that proliferating keratinocytes located at the edge of such colonies still contained nuclear YAP and little α-catenin at cell-cell junctions (at arrows). These data are consistent with the notion that in murine epidermis α-catenin negatively regulates YAP activity by modulating its interaction with 14-3-3 proteins, particularly at high cell densities (Schlegelmilch et al., 2011; Zhao et al., 2007).

The expression of YAP in stem cells and TA progenitors was then investigated at a clonal level (STAR Methods; Figure S1). We analyzed 160 clones from 5 different donors. Clonogenic keratinocytes gave rise to 12.2% ± 3.85%, 64.4% ± 6.94%, and 23.3% ± 3.33% (n = 5, mean ± SD) holoclones, meroclones, and paraclones, respectively. Clonal conversion, namely, the transition from holoclones to paraclones, was marked by progressive decrease of p63α, YAP, and TAZ (Figure 1D). Strikingly, nuclear YAP was detected in holoclones but was virtually undetectable in meroclones and paraclones (Figure 1E), while cytoplasmic, phosphorylated-YAP (P-YAPSer127) was barely detectable in holoclones and progressively increased in meroclones and paraclones (Figure 1F). Of note, survivin, a YAP target inhibiting anoikis in epidermal cells, was abundantly expressed

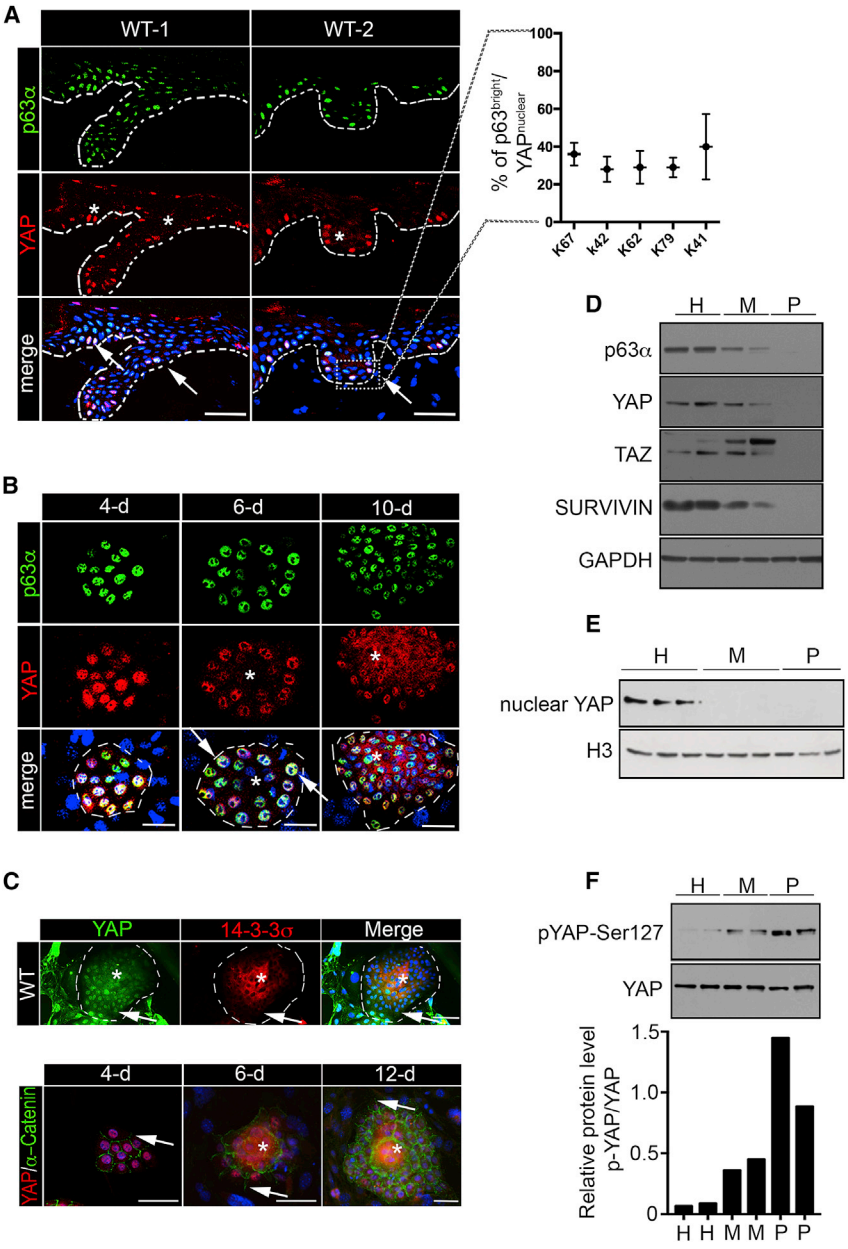


Figure 1. Expression of YAP in Normal Human Keratinocytes

(A) Immunofluorescence analysis of p63α and YAP expression in 7-μm-thick skin sections prepared from healthy donors (WT-1 and WT-2). Nuclear YAP is contained in basal keratinocytes expressing high levels of p63α (arrows). The inset shows the percentage of $p63\alpha^{bright}/YAP^{nuclear}$ cells in the epidermal basal layer (n = 5 WT donors, age 22–79 years). The $p63\alpha^{dim}$ and suprabasal keratinocytes contain cytoplasmic YAP (asterisk). Nuclei are stained with DAPI. Dotted line marks the epidermal-dermal junction. Scale bars, 40 μm.

(B) Immunofluorescence analysis of p63α and YAP in holoclone-type colonies derived from healthy donors at 4, 6, and 10 days of cultivation. The 4-day colonies are formed mainly by $p63\alpha^{bright}/YAP^{nuclear}$ cells. In 6- to 10-day colonies, nuclear YAP is contained in $p63\alpha^{bright}$ cells (arrows) at the edge the colonies, while the central part of such progressively growing colonies is formed mainly by keratinocytes containing cytoplasmic YAP (asterisk). Dotted line marks the colony perimeter. Scale bars, 20 μm.

(C) Top: normal keratinocytes (WT) in the central part of 10-day colonies contain cytoplasmic YAP and 14-3-3σ (asterisks), which is not detected in $YAP^{nuclear}$ cells located at the edge of the colonies (arrows). Bottom: $YAP^{nuclear}$ cells express low amounts of α-catenin located at cell-cell junctions (arrows). Cells located at the center of growing 6- to 12-day colonies express high levels of α-catenin and contain only cytoplasmic YAP. Scale bars, 20 μm (4-6 days); 10 μm (12 days).

(D) Western analysis of total cell extracts from cultures generated by holoclones (H), meroclones (M), and paraclones (P) isolated by clonal analysis (see STAR Methods and Figure S1) of sub-confluent normal human primary keratinocyte cultures. Clonal conversion is marked by progressive decrease of p63α, YAP and TAZ, and survivin (YAP target).

(E) Western analysis of nuclear cell extracts shows that nuclear YAP is detected exclusively in holoclones (H). Histone H3 was used as loading control.

(F) Top: western analysis of total cell extracts from cultures generated by H, M, and P shows that clonal conversion is marked by progressive increase of serine127-phosphorylated (inactive) YAP. Bottom: the pYAP/YAP ratio observed during clonal conversion. Total YAP was used as loading control.

in holoclones, barely detectable in meroclones, and undetectable in paraclones, hence mirroring the expression of nuclear YAP (Figures 1D and 1E).

YAP Sustains Epidermal Stem Cells and Regulates Clonal Conversion

We ablated YAP and TAZ in short- and long-term assays by means of specific small interfering RNA (siRNA) and short hairpin RNA (shRNA) (STAR Methods). In short-term (48 h) assays, neither siRNA-YAP nor siRNA-TAZ was able to significantly modulate the expression of p63α, although survivin slightly decreased in cells exposed to siRNA-YAP. Strikingly, simultaneous ablation of YAP and TAZ completely abolished the expression of both p63α and survivin (Figure S2A).

Primary keratinocytes were stably transduced with a lentiviral vector carrying specific YAP microRNA-adapted shRNA under a Tet-promoter and TAZ microRNA-adapted shRNA under a hCMV-promoter (STAR Methods). As compared to mock-transduced cells ($shCtr_{1/2}$), YAP short hairpin RNA (shYAP) and TAZ short hairpin RNA (shTAZ) reduced the expression of YAP and TAZ of ~90% and ~70%, respectively (Figure 2A). Note that downregulation of YAP and TAZ induced a mutual compensating increase of the paralog, more evident with shYAP (Figure 2A). As with siRNA-YAP and -TAZ (Figure S2A), these mutual offsetting

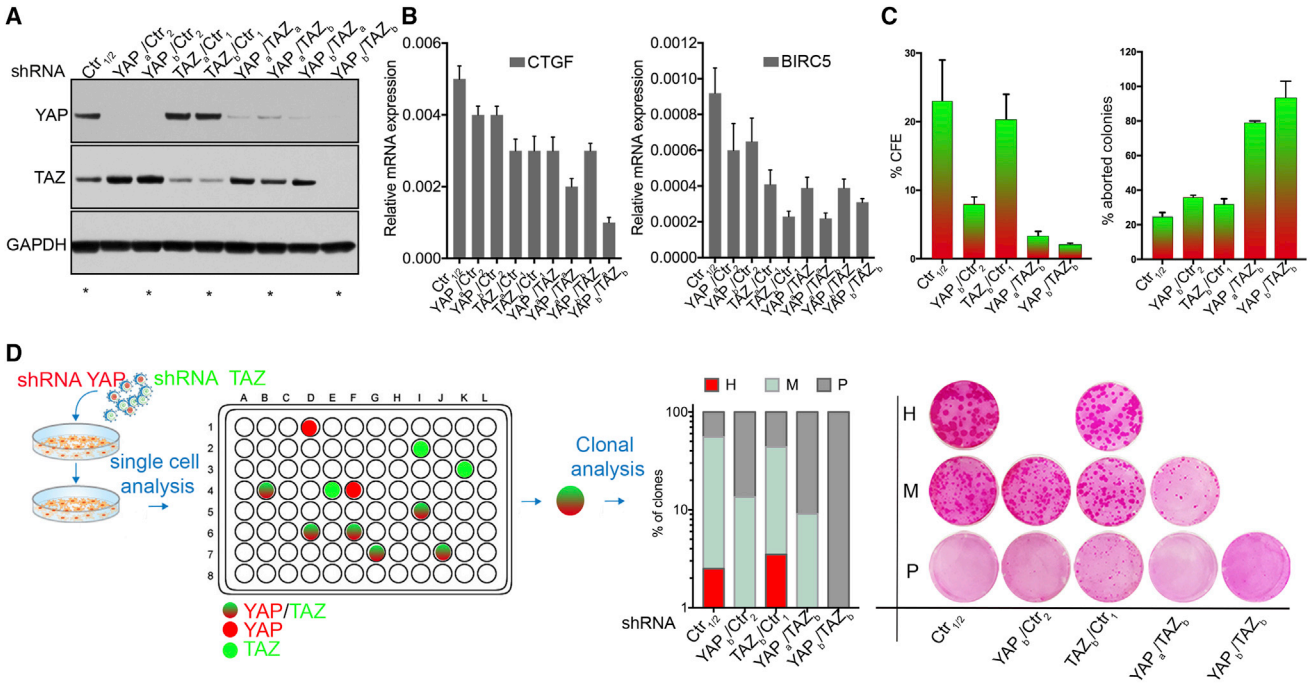


Figure 2. Downregulation of YAP and TAZ

(A) Western blot was performed on total protein extracts isolated from normal human keratinocytes transduced with TRIPZ-inducible lentiviral shRNA ($YAP_{a/b}$ and Ctr_1) and GIPZ-lentiviral shRNA ($TAZ_{a/b}$ and Ctr_2). Control cells were co-transduced with Ctr_1 and Ctr_2. Asterisks indicate samples used for further analysis. Doxycycline (0.5 μg/ml) was used to induce shRNA expression.

(B) qRT-PCR on mRNAs obtained from keratinocytes transduced with different shRNAs was used to determine the expression of 2 different YAP target genes (*CTGF* and *BIRC5*) (n = 3; mean ± SD). GAPDH mRNA was used to normalize the RT-PCR.

(C) Colony forming efficiency (CFE; left) and percentage of aborted colonies (right) were evaluated on keratinocytes transduced with different shRNA (as indicated). Doxycycline (0.5 μg/ml) was added 24h after transduction. Assays were performed at the first passage (7 d after transduction) (n = 2; mean ± SD).

(D) Left: clonal analysis of shRNA transduced clonogenic keratinocytes (see STAR Methods). Only the double positive clones (shCtr1/shCtr2, shYAP/shCtr2, shTAZ/shCtr1, and shYAP/shTAZ) were selected for clonal analysis. Middle: note that holoclones (H, red) could not be detected in shYAP transduced cultures, which still generated meroclones (M, light blue) and paraclones (P, gray). Keratinocytes co-transduced with shYAP and shTAZ generated virtually only paraclones. Right: representative cultures generated by these transduced clones.

effects explain the almost normal expression of TAZ in many double shYAP/shTAZ-transduced cultures (Figure 2A). Further experiments were thus performed with specific shYAP and shTAZ (Figure 2A, asterisks), which determined significant ablation of both YAP and TAZ and repression of their target genes *CTGF* and *BIRC5* (survivin) (Figures 2A and 2B).

As shown in Figure 2C (left panel), shCtr- and shTAZ-transduced cultures had a similar colony forming efficiency (CFE), whereas shYAP caused an over 50% decrease of clonogenic cells. But neither shRNAs significantly fostered clonal conversion, as indicated by a similar percentage of aborted colonies observed in shCtr, shYAP, and shTAZ (Figure 2C, right panel). Strikingly, simultaneous ablation of YAP and TAZ dramatically decreased CFE and generated only aborted colonies (Figure 2C). Thus, the clonogenic ability of YAP- and TAZ-ablated cells vanished in a single cell passage, and cells underwent growth arrest (see also Figure S2B).

Clonal analysis was then performed on primary cultures transduced with (1) $shCtr_1/shCtr_2$, (2) $shCtr_2$/shYAP, (3) $shCtr_1$/ShTAZ, and (4) shYAP/shTAZ (STAR Methods). Colonies were identified by Turbo-RFP ($shCtr_1$ and shYAP) and Turbo-EGFP ($shCtr_2$/shTAZ). A total of 262 double-colored clones were analyzed: 83 clones were transduced with $shCtr_{1/2}$; 52 and 59 clones were transduced with $shCtr_2$/shYAP or $shCtr_1$/shTAZ, respectively; and 68 clones were transduced with shYAP/shTAZ (Figure 2D, left). Each clone was transferred to an indicator dish for the classification of clonal type (Figure 2D, left; see also STAR Methods). As shown in Figure 2D (middle), a similar percentage of holoclones, meroclones, and paraclones was detected in $shCtr_{1/2}$ and shTAZ-transduced cells. Strikingly, ablation of YAP induced the selective disappearance of holoclones and a significant decrease of meroclones and increase of paraclones. Keratinocytes transduced both with shYAP and shTAZ generated virtually only paraclones. Figure 2D (right panel) shows representative cultures generated by these transduced clones. These data confirm that simultaneous YAP and TAZ ablation totally abolishes keratinocyte clonogenic ability and points to a critical role of YAP in upholding human epidermal stem cells.

Primary keratinocytes were then transduced with lentiviral vectors expressing Tet-inducible FLAG-YAP constructs, namely, YAP, YAP5SA (a constitutively active YAP), YAPS127A (carrying alanine substitution for Ser127, which abolishes the

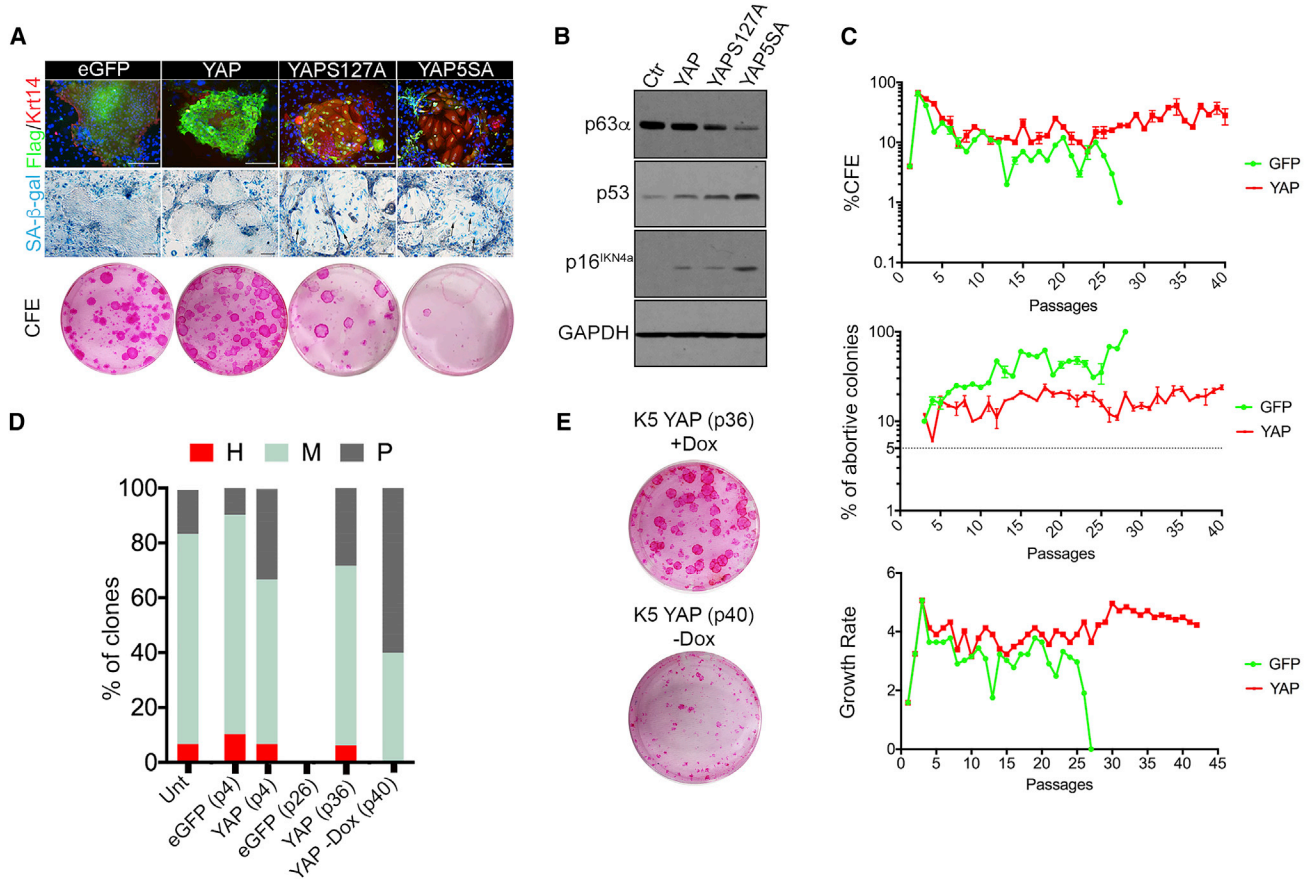


Figure 3. Enforced Expression of YAP and YAP Mutants

(A) Top: primary epidermal cultures were transduced with Tet-inducible lentiviral vectors carrying EGFP or the indicated FLAG-YAP constructs. Sub-confluent cultures were stained with FLAG-specific and keratin 14 (Krt14) antibodies. Middle: the same cultures were stained for β-galactosidase. Bottom: their colony forming efficiency (CFE) was determined.

(B) Western analysis of total cell extracts from YAPS127A- and YAP5SA-transduced cultures shows that premature replicative senescence was confirmed by upregulation of p53 and $p16^{INK4a}$ (and downregulation of p63α).

(C) Serial cultivation of EGFP- (green) and YAP-transduced (red) epidermal cultures. Top: CFE values are expressed as the ratio of the total number of colonies on the number of inoculated cells. All colonies were scored whether progressively growing or aborted. Middle: percentage of aborted colonies on the total number of colonies. Bottom: growth rate was calculated as population doublings per passage (see STAR Methods). YAP-transduced cultures show bypass of replicative senescence and indefinite proliferative potential.

(D and E) Clonal analysis of EGFP- and YAP-transduced epidermal cultures. The percentage of holoclones (H, red), meroclones (M, light blue), and paraclone (P, gray) has been calculated at the indicated cell passages (p). In YAP-transduced cells, the percentage of holoclones and the H/M/P ratio remained stable even at p36, while no clones were detected at p26 in EGFP-transduced cells. The block of clonal conversion was abolished upon removal of Doxycyclin (at p36), and YAP-transduced cultures lost their holoclones (D) as well as their clonogenic ability (E) after 4 passages.

interaction of YAP with 14-3-3σ), and YAPS94A (a mutant unable to bind TEAD transcription factors).

Although enforced YAP remained largely confined to the cytoplasm (Figure 3A, upper panel), the fraction of exogenous YAP able to move to the nucleus was transcriptionally active (Figure S3A). YAP induced a small increase of the keratinocyte clonogenic ability (Figures 3A, lower panel, and S3B); yet, it dramatically boosted their proliferative potential. The EGFP-transduced keratinocytes underwent 180 cell generations before senescence, which occurred after 27 cell passages. In contrast, YAP-transduced keratinocytes readily bypassed replicative senescence and continued to divide indefinitely. To date, they underwent 268 cell doublings and have been passaged 42 times.

Of note, serial cultivation of YAP-transduced cells clearly demonstrated that the extension of their lifespan (or even potential immortalization) was not marked by a progressive increase of the clonogenic ability or a progressive decrease of aborted colonies. Indeed, YAP-transduced keratinocytes at early and late cell passages had similar CFE (Figure 3C, upper panel) and contained a similar number of aborted colonies (Figure 3C, middle panel). Furthermore, YAP did not increase keratinocyte growth rate, which remained unchanged during the long serial cultivation and was comparable to that of early passages of EGFP-transduced cells (Figure 3C, lower panel). Strikingly, the initial percentage of holoclones, meroclones, and paraclones remained unchanged in YAP-transduced keratinocytes at p4

and p36 (Figure 3D). In contrast, clonal conversion occurred in EGFP-transduced cells, and no EGFP colonies could be detected already at p26 (Figure 3D).

These data show that YAP regulates neither keratinocyte growth rate nor their clonogenic ability. YAP halts clonal conversion, forcing keratinocytes into the stem cell compartment, hence extending indefinitely their proliferative potential. This blockage is indeed reversible, inasmuch as removal of doxycycline was marked by the progressive disappearance of holoclones (and meroclones) (Figures 3D and 3E), leading to a senescent culture containing virtually only aborted colonies, as with EGFP-transduced cultures.

Extending keratinocyte lifespan without altering the proportion of the different clonal types (Figure 3D) strongly suggests that YAP does not induce clonal reversion and hence does not revert an epidermal progenitor into a stem cell. This notion was confirmed by enforcing YAP into clonally isolated meroclones and paraclones (Figure S3D, upper panel). Both short-term (1 week) and constitutive overexpressions of YAP neither increased the proliferative potential of meroclones and paraclones (Figure S3D) nor halted their clonal conversion, inasmuch as the percentage of aborted colonies progressively increased both in the presence and in the absence of doxycycline (Figure S3E) and never reached the value of 5% (Figure S3E, dot lines) that defines holoclones (Figure S1).

These findings strengthen the notion that YAP specifically sustains holoclones, halting their clonal conversion and fostering their self-renewal. YAP executes these biological effects through canonical TEAD transcription factors, as keratinocytes transduced with the YAPS94A reduced their clonogenic ability (Figure S3B) and showed an increase of aborted colonies and downregulation of p63α and survivin (Figure S3C). This phenotype suggests a dominant-negative effect of enforced YAPS94A on YAP in keratinocytes, as reported in other cell types.

In sharp contrast to YAP, constitutively active YAP5SA and YAPS127A had a catastrophic effect on keratinocytes and induced their almost-immediate growth arrest (Figures 3A and S3F). Both mutants were located in the nuclei (Figure 3A, upper panel), potently induced YAP target genes (Figure S3A) and a strong decrease of clonogenic ability, which was dramatic in YAP5SA-transduced cells (Figure 3A, lower panel). Both YAP5SA- and YAPS127A-transduced keratinocytes displayed hallmarks of premature replicative senescence, which is envisaged as a potent tumor-suppressive mechanism that can be triggered in response to aggressive mitogenic stimuli or oncogenes (Serrano et al., 1997). Indeed, YAP5SA- and YAPS127A-transduced keratinocytes increased their size, expressed β-galactosidase (Figure 3A middle panel), and overexpressed $p16^{INK4a}$ and p53 (Figure 3B).

YAP Dysregulation and Stem Cell Loss in JEB

The notion that basal clonogenic keratinocytes adhere to the basement membrane and their intermediate filaments contribute to hemidesmosomes prompted us to investigate whether laminin 332-dependent adhesion defects could impinge on the YAP pathway, hence explaining stem cell loss and/or rapid clonal conversion of JEB cultures.

Two RDEB patients (9- and 12-year-olds) and 5 patients with generalized JEB (STAR Methods) were included in this study. JEB-1, JEB-4, and JEB-5 were 37, 49, and 26 years old, respectively, while JEB-2 and JEB-3 were 7 years old and 3 months old, respectively. Type VII collagen and laminin 332 were highly reduced in RDEB and JEB patients, respectively (see Bauer et al., 2017; Hirsch et al., 2017; Mavilio et al., 2006; and Figure S4A). Because of limited access to patients' specimens, not all experiments reported hereafter were performed on samples obtained from all 5 JEB patients.

In normal skin, nuclear YAP was expressed almost exclusively in $p63\alpha^{bright}$ basal keratinocytes (Figure 4A). In contrast, basal JEB keratinocytes contained mostly cytoplasmic YAP (Figure 4A). Of note, $p63\alpha^{bright}$ keratinocytes were still present in infants' skin sections (JEB-3), less represented in children (JEB-2), and barely detectable in adult patients (JEB-1).

YAP, TAZ, p63α, and survivin were dramatically decreased in JEB, but not RDEB, primary cultures (Figure 4B). As observed in normal mero and paraclones (Figure 1F), JEB cultures contained mostly phosphorylated YAP (Figure 4B). JEB cultures also contained $p16^{INK4a}$, which was barely detectable in normal and RDEB cultures (Figure 4B), suggesting a premature clonal conversion. Normal and RDEB colonies contained a mixed population of $YAP^{nuclear}/p63\alpha^{bright}$ and $YAP^{cytoplasmic}/p63\alpha^{dim/null}$ cells (Figure 4C, wild type [WT] and RDEB), while JEB colonies were formed mostly by keratinocytes containing cytoplasmic YAP (Figure 4C, JEB-3 and S4B). Similarly to what was observed in skin sections, some residual $p63\alpha^{bright}$ keratinocytes were still present in cultures from JEB infants (Figure 4C, JEB-3), but they progressively decreased in colonies generated from JEB children and adults (Figure S4B, JEB-2 and JEB-1). Accordingly, normal and RDEB cultures had comparable proliferative potential, which was dramatically decreased in JEB cultures (Figure 4D).

The residual proliferative capability of JEB cultures (Figure 4D) is highly reminiscent of the proliferative potential typically observed in clonally isolated meroclones and paraclones (Pellegrini et al., 1999a) (see also Figure S3D), strongly suggesting a selective loss of holoclone-forming cells. Indeed, clonal analysis showed that the small percentage of holoclones still detected in primary JEB-2 cultures vanished in a few passages (Figure 4E). Very few holoclone-forming cells could be detected in JEB-1, but only after multiple clonal analyses (Mavilio et al., 2006). Of note, JEB cultures unveiled a dramatic decrease of β4, but not of β1, integrins (Figure 4F), even if both integrins can act as laminin 332 receptors.

Thus, JEB is marked by a progressive depletion of holoclone-forming cells, phenocopying YAP-ablated normal keratinocytes. JEB features were recapitulated by short-term downregulation of the β3 chain of laminin 332 in normal keratinocytes by means of specific siRNA. As shown in Figure S4C, three different siRNAβ3s rapidly abolished the expression of YAP, p63α, and survivin, and cells contained almost exclusively cytoplasmic P-YAP. Of note, TAZ increased in such cells, supporting the notion that downregulation of YAP induces a compensating increase of the paralog (Figure S4C). However, TAZ did not prevent siRNAβ3-induced downregulation of p63α (Figure S4C).

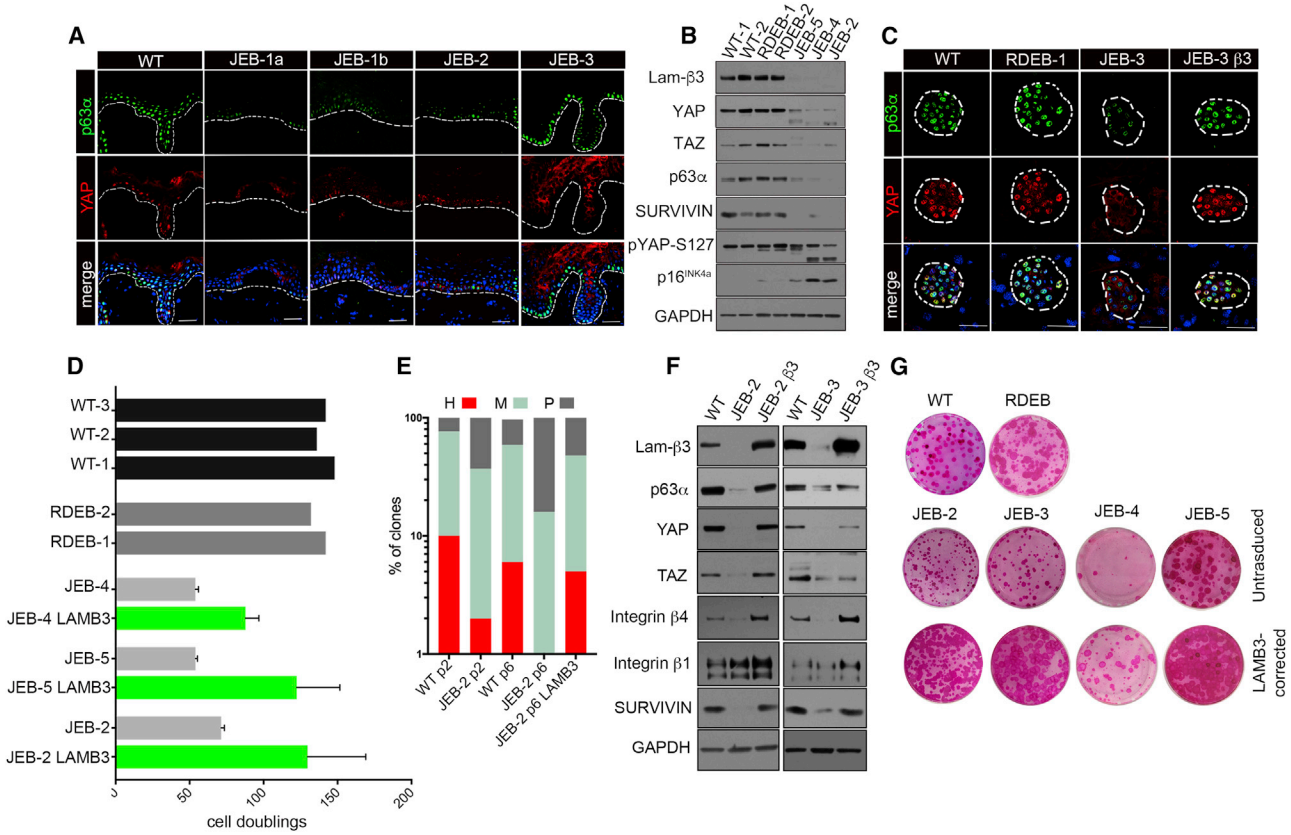


Figure 4. YAP Dysregulation in JEB

(A) Immunofluorescence analysis of p63α and YAP expression in 7-μm-thick skin sections prepared from normal skin (WT) and *LAMB3*-deficient JEB patients (see STAR Methods for patients' details). Low expression of cytoplasmic YAP marks JEB keratinocytes. DAPI (blue) stains nuclei. Dotted line marks the epidermal-dermal junction. Scale bars, 40 μm.

(B) Western analysis of total cell extracts from WT, RDEB, and JEB cultures immunostained with indicated antibodies.

(C) Immunofluorescence analysis of p63α and YAP in 5-day primary colonies derived from healthy donors (WT) and RDEB-1 and JEB-3 patients. JEB-3-*LAMB3* shows colonies initiated after gene therapy of JEB-3 cultures (cells transduced with a γRV vector carrying a wild-type *LAMB3* cDNA, see main text). Scale bars, 10 μm.

(D) Serial cultivation and calculation of cumulative cell doublings in WT (black), RDEB (gray), JEB (light gray), and *LAMB3*-corrected JEB (green) cultures. The number of cell doublings was calculated as described in STAR Methods (JEB-4, n = 3; JEB-5, n = 2, JEB2, n = 2, mean ± SD).

(E) Clonal analysis of WT, JEB, and *LAMB3*-corrected JEB cultures. The percentage of holoclones (H, red), meroclones (M, light blue), and paraclone (P, gray) has been calculated at the indicated cell passages (p).

(F) Western analysis of total cell extracts from WT, JEB, and *LAMB3*-corrected JEB cultures immunostained with indicated antobodies (similar results were obtained with other JEB cultures).

(G) CFE of keratinocyte cultures initiated from normal skin (WT) and RDEB and JEB patients before (control [CTR]) and after genetic correction (*LAMB3*).

Gene Therapy Restores Functional YAP and Rescues Epidermal Stem Cells

Primary JEB keratinocytes were transduced with clinical-grade gamma retroviral vectors (γRV) expressing full-length *LAMB3* cDNA under the control of the Moloney leukemia virus long terminal repeat. Transduction efficiency of clonogenic cells was over 95%, as indicated by immunofluorescence analysis of transgenic colonies (Bauer et al. 2017; Hirsch et al., 2017; Mavilio et al., 2006). *LAMB3*-transduced JEB cultures expressed normal levels of laminin 332-β3 (Figure 4F). Genetic correction of JEB keratinocytes induced (1) expression of YAP and TAZ, p63α, and survivin (Figure 4F); (2) nuclear translocation of YAP and its co-localization with p63α (Figure 4C, *LAMB3*-JEB-3 and S4B); (3) restoration of β4 integrins (Figure 4F); and (4) restoration of keratinocyte clonogenic ability (Figure 4G) and proliferative potential (Figure 4D). All these parameters were comparable to those of normal keratinocytes. Finally, *LAMB3*-gene therapy rescued JEB epidermal stem cells when the transduction occurred at early cell passages (p2). As shown in Figure 4E, the few holoclones present in primary JEB-2 cultures were lost already in few passages. In contrast, *LAMB3*-corrected cultures preserved a percentage of holoclone-forming cells comparable to that of a normal control during serial cultivation (Figure 4E).

Enforced YAP Recapitulates Gene Therapy of *LAMB3*-JEB and Decouples Cell Adhesion from Stemness

Enforced YAP and *LAMB3*-gene were equivalent in restoring expression of p63α and survivin (Figure 5A), clonogenic ability

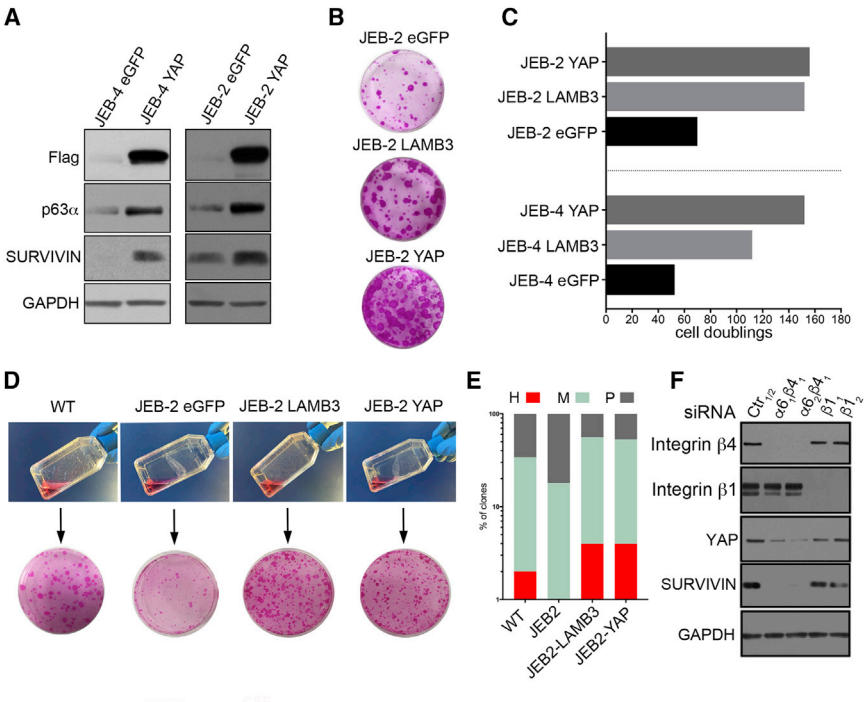


Figure 5. Enforced YAP Recapitulates *LAMB3* Gene Therapy

(A) Western analysis of total cell extracts from Tet-inducible EGFP and FLAG-YAP transduced JEB cultures immunostained with indicated antibodies.

(B and C) CFE (B) and proliferative potential (C) of JEB keratinocytes transduced with EGFP, *LAMB3*, and YAP. The CFE was plated at a density of 1000 cells per dish, and colonies were stained with Rhodamine B 12 days later. The number of cell doublings was calculated as described in STAR Methods.

(D) Top: adhesion of confluent cultured epidermal sheets prepared from normal keratinocytes (WT) and JEB cultures transduced with Tet-inducible EGFP, *LAMB3*, and YAP. The mere transportation of the flask from the incubator to the hood caused the spontaneous detachment of JEB and JEB-YAP cultures (arrows), while *LAMB3*-corrected keratinocytes remained firmly attached to the substrate (asterisks). Bottom: CFE performed on the above cultures. The CFE was plated at a density of 2.000 cells per dish, and colonies were stained with Rhodamine B 12 days later.

(E) Clonal analysis of the cultures of (D). WT, JEB, and *LAMB3*-corrected JEB cultures. The percentage of holoclones (H, red), meroclones (M, light blue), and paraclone (P, gray) has been determined. Note that enforced YAP in a *LAMB3* null background prevents the stem cell loss, even in the absence of cell adhesion.

(F) Western analysis of total cell extracts from normal keratinocytes co-transfected with the indicated combinations of siRNA for α6, β4, and β1 integrins (see Table S3) and immunostained with indicated antibodies.

(Figure 5B), and proliferative potential (Figure 5C) of JEB keratinocytes.

Of note, YAP-mediated effects occurred even in the presence of defective adhesion. Normal keratinocyte colonies eventually fuse, giving rise to a cohesive epidermal sheet that remains firmly attached to the vessel (Figure 5D, WT), from which it can only be released after prolonged (30–40 min) enzymatic treatment with the neutral proteases Dispase II (Gallico et al., 1984; Green et al., 1979; Hirsch et al., 2017; Pellegrini et al., 1999b). Dispase II can detach *in situ* epidermis from the underlying dermis as well. In contrast, epidermal sheets generated by JEB colonies spontaneously detached from the culture vessel (even during the mere transportation from the incubator to the hood) and lost their clonogenic ability (Figure 5D, EGFP). Thus, as with normal skin, the basal layer of fully differentiated cultured epidermis requires the interaction of basal keratinocytes with properly assembled heterotrimeric laminin 332. Indeed, when *LAMB3*-corrected JEB keratinocytes became confluent and stratified, the resulting epidermal sheet remained firmly attached to the culture vessel, required prolonged Dispase II treatment for detachment and maintained a clonogenic ability similar to that of a normal control (Figure 5D, *LAMB3*). Enforced YAP did not restore the adhesive properties of JEB epidermal sheets but fully preserved their clonogenic ability (Figure 5D, YAP).

Strikingly, enforced YAP sustained JEB holoclone-forming cells to a similar extent of *LAMB3*-gene therapy (Figure 5E) even after epidermal detachment, thus decoupling epidermal adhesion from stemness.

Laminin 332, Integrins, and YAP in JEB

The notions that (1) JEB colonies, if established, are able to grow and reach confluence; (2) confluent JEB epidermal sheets spontaneously detach from the vessel and lose their clonogenic ability at once; and (3) enforced YAP can preserve clonogenic cells of confluent sheets in the absence of adhesion confirm that growing keratinocyte colonies, mimicking wound healing rather than epidermal homeostasis, use a different adhesion machinery than that used by fully mature epidermis (DiPersio et al., 2016). For instance, human keratinocytes use mostly β1 integrins during initial adhesion, migration, and growth, while α6β4 integrins are uniquely located in hemidesmosomes and mediate the firm adhesion of basal keratinocytes to the basement membrane (Borradori et al., 1997; De Luca et al., 1990; Hopkinson and Jones, 2000; Rousselle et al., 1997; Sonnenberg et al., 1991; Stepp et al., 1990). Indeed, mutations in *ITGA6* and *ITGB4* (encoding α6β4), but not *ITGB1* (encoding β1 integrins), give rise to a form of JEB virtually indistinguishable from *LAMB3*-deficient JEB (Fine et al., 2008).

This prompted us to investigate whether YAP expression was modulated by β1 or β4 integrins, both of which are able to bind laminin 332 (DiPersio et al., 2016; Rousselle and Beck, 2013). Laminin 332-deficient JEB is marked by a concomitant downregulation of α6β4 (Figure 4F; De Rosa et al., 2013; Hirsch et al., 2017) due to both reduced transcription and increased protein degradation (Matsui et al., 1998; McMillan et al., 1997; Ryan et al., 1999). Such α6β4 downregulation was not accompanied by a concomitant downregulation of β1 integrins (Figure 4F). In

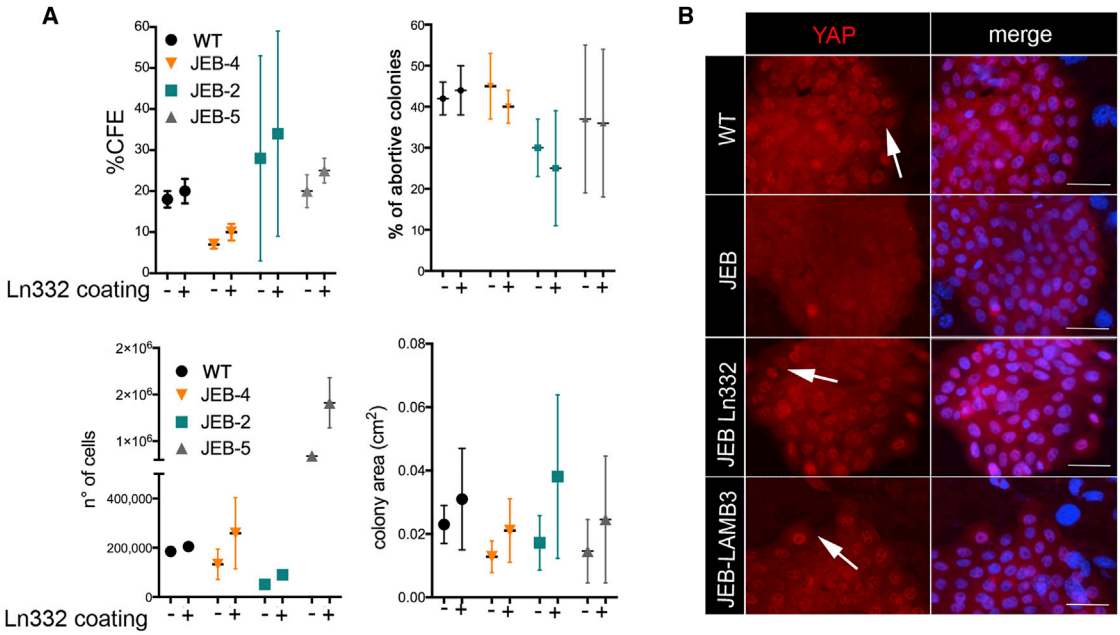


Figure 6. Laminin 332 Supports JEB Cell Growth and Clonogenic Properties

(A) WT and JEB cells were inoculated on uncoated and laminin 332-coated dishes (2.5 μg/ml) (500−1000 cells per dish) (see STAR Methods). Top left: CFE values are expressed as the ratio of the total number of colonies on the number of inoculated cells. Top right: percentage of aborted colonies on the total number of colonies. Bottom left: number of cells recovered after 12 days of cultivation. Bottom right: mean of the area of the colonies (n = 3, mean ± SD).

(B) Immunofluorescence analysis of YAP in WT, JEB, and *LAMB3*-corrected JEB cells plated on uncoated dishes and of JEB keratinocytes plated on laminin 332-coated dishes (JEB-Ln332). Note the nuclear localization of YAP in cells at the edge of the colonies, both in laminin 332-coated dishes and in *LAMB3*-corrected cells (arrows). DAPI (blue) stains nuclei.

normal keratinocytes, siRNA-mediated downregulation of α6β4, but not β1, integrins induced a downregulation of YAP and its targets (Figures 5F and S5A). Strikingly, downregulation of β1 integrins induced a concomitant upregulation of α6, which determined a concomitant increase of the expression of YAP and its target genes in the presence of normal expression of laminin 332 (Figure S5A). Taken together, these findings show that the interaction of laminin 332 with α6β4, but not β1, integrins is instrumental for the function of YAP both in JEB and in normal epidermal homeostasis and strengthen the notion that migrating keratinocytes can use β1 integrins for their interaction with collagen, fibronectin, and other cell-derived extracellular matrix proteins independently of YAP (see Rousselle and Beck, 2013 for review).

Based on this notion, we investigated whether cultivation of primary JEB epidermal cells could be improved by coating culture vessels with recombinant laminin 332. As shown in Figure 6A (upper panels), no significant difference was detected in the number of clonogenic cells and aborted colonies both in normal and in JEB keratinocytes grown on laminin 332. However, both colony size and cell yield were significantly higher when JEB keratinocytes were cultured on laminin 332-coated vessels (Figure 6A, lower panels). As expected, JEB keratinocytes located at the edge of colonies cultured on coated vessels contained nuclear YAP, which was instead undetectable in cells grown on uncoated dishes (Figure 6B). Of note, normal keratinocytes cultured on coated dishes generated larger colonies but not a higher number of cells (Figure 6A, lower panels).

Combined *Ex Vivo* Cell and Gene Therapy Restores YAP Function *In Vivo*

JEB has been tackled by combined *ex vivo* cell and gene therapy. Three patients were treated with autologous epidermal cultures transduced with a γRV expressing full-length *LAMB3* cDNA (Bauer et al., 2017; De Rosa et al., 2013; Hirsch et al., 2017; Mavilio et al., 2006).

At admission, basal keratinocytes of these patients contained barely detectable amounts of β3-laminin 332 and p63α and virtually only cytoplasmic YAP (Figure 7). At 3–12 years after transplantation of transgenic epidermal cultures, the regenerated epidermis was normal-looking, remained mechanically stable throughout the entire follow-up period, and did not form blisters, even upon shear force. We observed a proper expression and location of laminin 332 in the basal lamina (refs and Figure 7). *In situ* hybridization performed using vector-specific *LAMB3* probes showed homogeneous expression of *LAMB3* mRNA in all epidermal layers, confirming that the regenerated epidermis consists only of transgenic keratinocytes (Bauer et al. 2017; De Rosa et al., 2013; Hirsch et al., 2017). Histological analysis showed a normal and fully differentiated epidermis with a normal dermal-epidermal junction. Electron Microscopy confirmed the presence of well-defined, organized hemidesmosomes comparable to those of healthy controls (Bauer et al. 2017; De Rosa

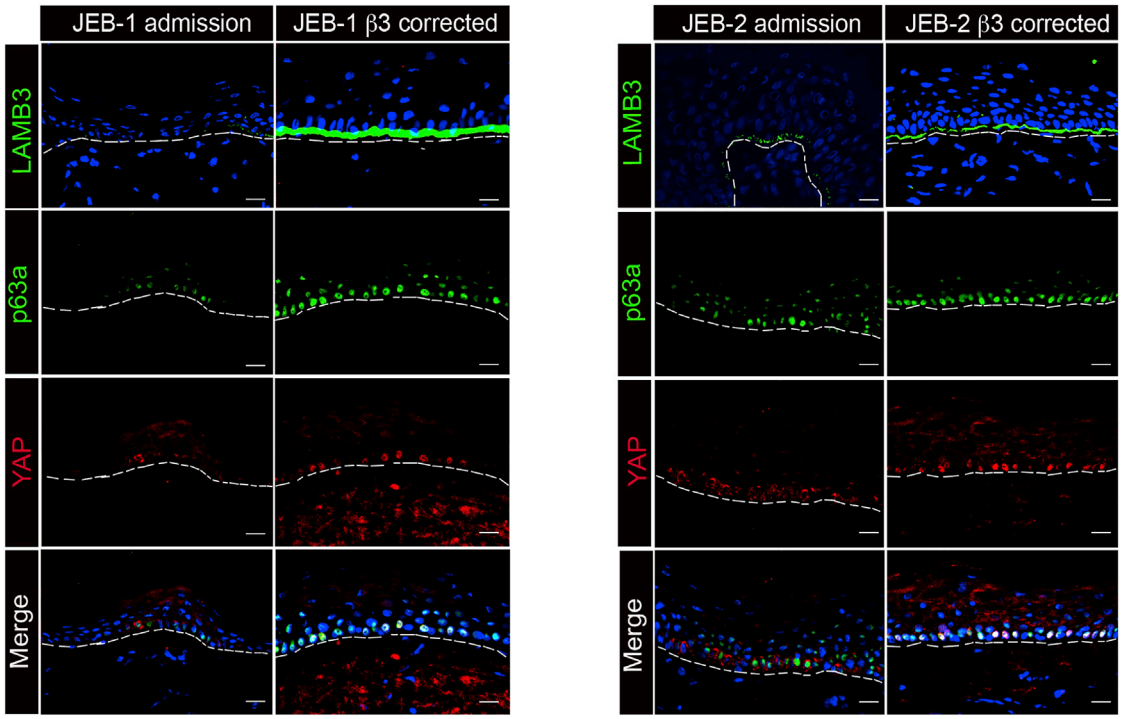


Figure 7. Combined Cell and Gene Therapy Rescues YAP Activity *In Vivo*

Immunofluorescence analysis of laminin 5 (β3 chain), p63α, and YAP expression in 7-μm-thick skin sections prepared from JEB-1 and JEB-2 patients before (admission) and after (6.5 years and 8 months, respectively) combined cell and gene therapy (β3 corrected; see De Rosa et al., 2013 and Hirsch et al., 2017 for details). Note restoration of the expression of p63α and YAP, both contained in the nuclei (stained with DAPI) of basal keratinocytes. Dotted line marks the epidermal–dermal junction. Scale bars, 20 μm.

et al., 2013; Hirsch et al., 2017). As shown in Figure 7, the basal layer of the newly formed transgenic epidermis contained a number of YAP^{nuclear}/p63α^{bright} cells similar to that of a normal skin (Figure 1A).

DISCUSSION

The boundary between the epidermis and the underlying dermis is established by the basement membrane. Polarized keratinocytes of the epidermal basal layer are anchored to the basement membrane by a variety of integrin receptors (Margadant et al., 2010; Watt, 2002). Particularly during cell migration and wound healing, β1 integrins provide transmembrane connections to the intracellular actin network through recruitment of several factors to their cytoplasmic tails (Simpson et al., 2011). Instead, α6β4 integrin has a unique role in organizing large adhesion complexes called hemidesmosomes. From one side, α6β4 is tethered to intermediate filaments by plectin and bullous pemphigoid antigens (BP180); from the other side, it binds to laminin 332, thereby linking keratins to collagen VII dermal anchoring fibrils. Of note, integrin complexes also transduce external cues into intracellular signals (Simpson et al., 2011). In doing so, junctional and cytoskeletal proteins control the maintenance of the stem cell population that resides in the epidermal basal layer.

JEB is due to mutations in genes coding for basically all the proteins forming hemidesmosomes, as laminin 332, α6β4 integrin, and collagen XVII (BP180). At variance with RDEB, laminin 332-dependent JEB is characterized by a depletion of stem cells (Mavilio et al., 2006). Here, we show that such depletion can be attributed to a dysregulation of the YAP and TAZ pathway. YAP-dependent preservation of epidermal stem cells requires integrity of the hemidesmosomal protein complex. Deleterious mutations of *LAMB3* and consequent laminin 332 ablation and disruption of its interaction with α6β4 lead to a dramatic decrease of YAP expression and to its translocation into the cytoplasm, thereby causing depletion of holoclone forming cells, which cannot be offset by TAZ. Accordingly, ablation of YAP induces the disappearance of holoclones in normal epidermal cultures. Our data explain the initial ability of JEB skin to heal the recurrent lesions and of established JEB colonies to proliferate, most likely through β1 integrins. The increase of CFE and cell yield observed when JEB keratinocytes were cultured on laminin 332-coated vessels is consistent with the notion that β1 integrin-mediated keratinocyte spreading, migration, and proliferation occurring during wound healing or cultivation still require laminin 332. Indeed, it has been reported that wounding of quiescent epidermis initiates adhesion and spreading of keratinocytes at the wound edge on endogenous basement membrane laminin 332 via both α3β1 and α6β4. The interaction of α6β4 with newly synthesized laminin 332 culminates in collagen digestion, laminin 332 deposition under the newly formed epidermis, and consequent hemidesmosome formation (Decline and Rousselle,

2001; Frank and Carter, 2004; Gonzales et al., 1999; Hamelers et al., 2005; Nguyen et al., 2000). Then, laminin 332 and its binding to α6β4 sustains the newly formed epidermis and its stem cell population, and YAP is instrumental in this process. Indeed, the development in the long term of chronic non-healing lesions is marked by stem cell depletion. Accordingly, mutations of α6β4, but not β1 integrins, cause a form of JEB virtually indistinguishable from *LAMB3*-deficient JEB (Fine et al., 2008).

Strikingly, enforced YAP can decouple adhesion from stemness, inasmuch as JEB keratinocytes overexpressing YAP maintain their stem cell content even in the absence of adhesion to the substrate.

Our data are consistent with the notion that YAP regulates murine and human epithelial stem cells (Barry et al., 2013; Schlegelmilch et al., 2011; Walko et al., 2017; Zhang et al., 2011; Zhao et al., 2007). In murine epidermis, α-catenin, a molecule sensing cell density in the skin, negatively controls YAP activity and phopsphorylation by modulating its interaction with 14-3-3σ (Schlegelmilch et al., 2011). In line with these findings, in human keratinocytes, nuclear YAP is contained in 14-3-3σ^{neg} α-catenin^{dim} cells located at the edge of growing colonies, while keratinocytes crowded in the central part of the colonies express abundant α-catenin and 14-3-3σ and contain only cytoplasmic, inactive P-YAP. Unraveling the role of YAP in regulating epidermal stem cells accounts for the intriguing observation that downregulation of 14-3-3σ forces human keratinocytes into the stem cell compartment and fosters immortalization of primary human epidermal cells without the need of exogenous oncogenes and/or oncoviruses (Dellambra et al., 2000). Accordingly, mice carrying a loss of function mutation in 14-3-3σ show unregulated nuclear YAP activity, epidermal progenitor expansion, and inhibition of differentiation (Sambandam et al., 2015).

When somatic cells register a preset number of generations, known as the Hayflick limit, they enter an irreversible state of growth arrest referred to as replicative senescence (Serrano et al., 1997). Clonal conversion and a precise cell-doubling clock control the timing of human keratinocyte replicative senescence (Barrandon and Green, 1987; Dellambra et al., 2000; Pellegrini et al., 1999a; Rochat et al., 1994). Serial propagation of keratinocytes is sustained by stem cells, and senescence occurs when the last stem cell present in culture loses its stemness and generates terminal colonies (Mathor et al., 1996). YAP halts clonal evolution and indefinitely preserves the original population of holoclone-forming cells but does not induce clonal reversion of meroclones into holoclone-forming cells. Thus, YAP is essential for self-renewal and proliferative potential of epidermal stem cells but does not induce clonal reversion of TA progenitors into bona fide stem cells.

YAP regulates identity, maintenance, and self-renewal of murine airway epithelial cells by functionally and genetically interacting with p63 (Zhao et al., 2014), a critical regulator of the proliferative potential of many epithelial stem cells (Senoo, 2013; Senoo et al., 2007). In line with these findings, in human keratinocytes, nuclear YAP strictly co-localizes with p63α^{bright} cells *in vivo* and proliferating p63α^{bright} cells located at the edge of growing colonies, while p63α$^{dim/neg}$ keratinocytes contain only cytoplasmic, inactive P-YAP.

Intriguingly, a dramatic difference exists between the expressions of P-YAP and p63a *in vivo* and *in vitro*, depending upon the age of the patient. The epidermal basal layer of all JEB patients contains only (or mostly) cytoplasmic YAP, irrespective of the age of the patient. Instead, basal p63α^{bright} keratinocytes are still present in JEB newborns, but they progressively disappear during the disease process and are still detectable in children and almost undetectable in adults. In contrast, all primary JEB cultures are formed by colonies containing P-YAP and low levels of p63α, which causes rapid clonal conversion and senescence.

This notion explains the hurdles encountered in the isolation and cultivation of JEB keratinocytes. While cultures can be relatively easily initiated from infants, multiple biopsies are usually required to initiate JEB cultures from adults. In such patients, successful cultures can be established only when biopsies are taken from body sites less affected by the disease. However, JEB epidermal cultures rapidly lose their stem cell content and undergo premature senescence irrespectively of the age of the patients and the donor body site. Thus, it is tantalizing to speculate that perhaps depending upon the type of mutation, there is a progressive, yet slow, YAP-dependent loss of JEB stem cells during the disease process, and such loss is dramatically accelerated during culture procedures. Accordingly, JEB patients slowly but progressively lose their ability to heal their continuously occurring skin blisters and eventually develop chronic non-healing wounds.

These findings have huge clinical implications. First, ideally, *ex vivo* gene therapy should be performed as soon as possible in children, before the exhaustion of stem cells occurring through the disease process. Second, to minimize the rapid clonal evolution and stem cells loss observed in culture, clonogenic keratinocytes should be transduced immediately after their isolation from the skin biopsy. When appropriately performed, *ex vivo* gene therapy can in fact prevent the rapid loss of JEB stem cells occurring during cultivation (refs and Figures 5 and 7). Third, the initial cultivation step should be performed on laminin 332 coated vessels to provide a laminin 332/α6β4 signal to JEB keratinocytes even before gene correction to further minimize the stem cell loss due to YAP dysregulation and to foster proliferation of the established clonogenic cells (Figure 6A). These data are supported by many lines of evidence on the use of laminin coating vessels to sustain self-renewal and stem cell properties in other cellular contexts (Rodin et al., 2010; Takayama et al., 2013).

In summary, our data show that signals emanating from laminin 332 at hemidesmosomes through α6β4 and not β1 underpin YAP activity, which is instrumental in sustaining human epidermal stem cells. *LAMB3*-dependent JEB triggers YAP inactivation and leads to epidermal stem cell depletion, supporting the notion that JEB is an adhesion and a stem cell disease. These findings will drive a better development of combined *ex vivo* cell and gene therapy for this devastating genetic skin disease.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING

- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Human tissues
  - Primary human cell cultures
  - Primary Junctional Epidermolysis Bullosa (JEB) and Dystrophic Epidermolysis Bullosa (RDEB) cell cultures
  - 3T3J2 cell line
  - MFG-LAMB3-Packaging cell line
- METHOD DETAILS
  - Generation of genetically corrected epidermal keratinocytes
  - Colony Forming Efficiency, population doublings and growth rate
  - Clonal analysis
  - Western blotting
  - Immunofluorescence
  - Senescence-Associated β-Galactosidase Staining
  - Plasmid constructs
  - Lentiviral production and primary human keratinocyte infection
  - Transient transfection
  - RNA extraction and real-time qPCR
  - Coating of Laminin 332
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistical analysis
  - Replications

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.04.055.

ACKNOWLEDGMENTS

This work was supported by the Italian Ministry of Education, University, and Research (MIUR) (CTN01_E48C13000140008) and Regione Emilia-Romagna, Por Fesr (E8IJ10000120007 and E92I16000220005). We thank Stefano Piccolo and Sirio Dupont for sharing reagents and plasmids.

AUTHOR CONTRIBUTIONS

L.D.R. and A.S.S., who contributed equally to this work, defined strategic procedures, performed the experiments, analyzed the data, assembled all input data, prepared the figures, and edited the manuscript. G.D.S. and G. Pellacani provided normal and RDEB human material used in all experiments. T.H., T.R., N.T., G. Pellegrini, and J.W.B. provided all JEB human materials and performed transplantation of the transgenic grafts, surgical and medical procedures, and clinical follow-up of some of the patients described in the paper. M.D.L. coordinated the study, defined strategic procedures, administered the experiments, and wrote the manuscript.

DECLARATION OF INTERESTS

G. Pellegrini and M.D.L. are co-founders and members of the Board of Directors of Holostem Terapie Avanzate (HTA), s.r.l, Modena, Italy, as well as consultants for J-TEC-Japan Tissue Engineering, Ltd.

Received: November 30, 2018
Revised: March 12, 2019
Accepted: April 10, 2019
Published: May 14, 2019

REFERENCES

Barrandon, Y., and Green, H. (1987). Three clonal types of keratinocyte with different capacities for multiplication. Proc. Natl. Acad. Sci. USA *84*, 2302–2306.

Barry, E.R., Morikawa, T., Butler, B.L., Shrestha, K., de la Rosa, R., Yan, K.S., Fuchs, C.S., Magness, S.T., Smits, R., Ogino, S., et al. (2013). Restriction of intestinal stem cell expansion and the regenerative response by YAP. Nature *493*, 106–110.

Bauer, J.W., Koller, J., Murauer, E.M., De Rosa, L., Enzo, E., Carulli, S., Bondanza, S., Recchia, A., Muss, W., Diem, A., et al. (2017). Closure of a Large Chronic Wound through Transplantation of Gene-Corrected Epidermal Stem Cells. J. Invest. Dermatol. *137*, 778–781.

Bisson, F., Rochefort, E., Lavoie, A., Larouche, D., Zaniolo, K., Simard-Bisson, C., Damour, O., Auger, F.A., Guérin, S.L., and Germain, L. (2013). Irradiated human dermal fibroblasts are as efficient as mouse fibroblasts as a feeder layer to improve human epidermal cell culture lifespan. Int. J. Mol. Sci. *14*, 4684–4704.

Borradori, L., Koch, P.J., Niessen, C.M., Erkeland, S., van Leusden, M.R., and Sonnenberg, A. (1997). The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the beta4 integrin subunit. J. Cell Biol. *136*, 1333–1347.

De Luca, M., D'Anna, F., Bondanza, S., Franzi, A.T., and Cancedda, R. (1988). Human epithelial cells induce human melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis. J. Cell Biol. *107*, 1919–1926.

De Luca, M., Tamura, R.N., Kajiji, S., Bondanza, S., Rossino, P., Cancedda, R., Marchisio, P.C., and Quaranta, V. (1990). Polarized integrin mediates human keratinocyte adhesion to basal lamina. Proc. Natl. Acad. Sci. USA *87*, 6888–6892.

De Rosa, L., Carulli, S., Cocchiarella, F., Quaglino, D., Enzo, E., Franchini, E., Giannetti, A., De Santis, G., Recchia, A., Pellegrini, G., and De Luca, M. (2013). Long-term stability and safety of transgenic cultured epidermal stem cells in gene therapy of junctional epidermolysis bullosa. Stem Cell Reports *2*, 1–8.

Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J., and Toussaint, O. (2009). Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nat. Protoc. *4*, 1798–1806.

Decline, F., and Rousselle, P. (2001). Keratinocyte migration requires alpha2-beta1 integrin-mediated interaction with the laminin 5 gamma2 chain. J. Cell Sci. *114*, 811–823.

Dellambra, E., Vailly, J., Pellegrini, G., Bondanza, S., Golisano, O., Macchia, C., Zambruno, G., Meneguzzi, G., and De Luca, M. (1998). Corrective transduction of human epidermal stem cells in laminin-5-dependent junctional epidermolysis bullosa. Hum. Gene Ther. *9*, 1359–1370.

Dellambra, E., Golisano, O., Bondanza, S., Siviero, E., Lacal, P., Molinari, M., D'Atri, S., and De Luca, M. (2000). Downregulation of 14-3-3sigma prevents clonal evolution and leads to immortalization of primary human keratinocytes. J. Cell Biol. *149*, 1117–1130.

Di Iorio, E., Barbaro, V., Ruzza, A., Ponzin, D., Pellegrini, G., and De Luca, M. (2005). Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc. Natl. Acad. Sci. USA *102*, 9523–9528.

DiPersio, C.M., Zheng, R., Kenney, J., and Van De Water, L. (2016). Integrin-mediated regulation of epidermal wound functions. Cell Tissue Res. *365*, 467–482.

Dobrokhotov, O., Samsonov, M., Sokabe, M., and Hirata, H. (2018). Mechanoregulation and pathology of YAP/TAZ via Hippo and non-Hippo mechanisms. Clin. Transl. Med. *7*, 23.

Dupont, S. (2016). Role of YAP/TAZ in cell-matrix adhesion-mediated signalling and mechanotransduction. Exp. Cell Res. *343*, 42–53.

Fine, J.D., Eady, R.A., Bauer, E.A., Bauer, J.W., Bruckner-Tuderman, L., Heagerty, A., Hintner, H., Hovnanian, A., Jonkman, M.F., Leigh, I., et al. (2008). The

classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB. J. Am. Acad. Dermatol. *58*, 931–950.

Fine, J.D., Bruckner-Tuderman, L., Eady, R.A., Bauer, E.A., Bauer, J.W., Has, C., Heagerty, A., Hintner, H., Hovnanian, A., Jonkman, M.F., et al. (2014). Inherited epidermolysis bullosa: updated recommendations on diagnosis and classification. J. Am. Acad. Dermatol. *70*, 1103–1126.

Frank, D.E., and Carter, W.G. (2004). Laminin 5 deposition regulates keratinocyte polarization and persistent migration. J. Cell Sci. *117*, 1351–1363.

Gallico, G.G., 3rd, O'Connor, N.E., Compton, C.C., Kehinde, O., and Green, H. (1984). Permanent coverage of large burn wounds with autologous cultured human epithelium. N. Engl. J. Med. *311*, 448–451.

Gonzales, M., Haan, K., Baker, S.E., Fitchmun, M., Todorov, I., Weitzman, S., and Jones, J.C. (1999). A cell signal pathway involving laminin-5, alpha3beta1 integrin, and mitogen-activated protein kinase can regulate epithelial cell proliferation. Mol. Biol. Cell *10*, 259–270.

Green, H., Kehinde, O., and Thomas, J. (1979). Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc. Natl. Acad. Sci. USA *76*, 5665–5668.

Hamelers, I.H., Olivo, C., Mertens, A.E., Pegtel, D.M., van der Kammen, R.A., Sonnenberg, A., and Collard, J.G. (2005). The Rac activator Tiam1 is required for (alpha)3(beta)1-mediated laminin-5 deposition, cell spreading, and cell migration. J. Cell Biol. *171*, 871–881.

Harvey, K.F., Zhang, X., and Thomas, D.M. (2013). The Hippo pathway and human cancer. Nat. Rev. Cancer *13*, 246–257.

Hirsch, T., Rothoeft, T., Teig, N., Bauer, J.W., Pellegrini, G., De Rosa, L., Scaglione, D., Reichelt, J., Klausegger, A., Kneisz, D., et al. (2017). Regeneration of the entire human epidermis using transgenic stem cells. Nature *551*, 327–332.

Hopkinson, S.B., and Jones, J.C. (2000). The N terminus of the transmembrane protein BP180 interacts with the N-terminal domain of BP230, thereby mediating keratin cytoskeleton anchorage to the cell surface at the site of the hemidesmosome. Mol. Biol. Cell *11*, 277–286.

Irvine, K.D., and Harvey, K.F. (2015). Control of organ growth by patterning and hippo signaling in Drosophila. Cold Spring Harb. Perspect. Biol. *7*, a019224.

Margadant, C., Charafeddine, R.A., and Sonnenberg, A. (2010). Unique and redundant functions of integrins in the epidermis. FASEB J. *24*, 4133–4152.

Markowitz, D., Goff, S., and Bank, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. Virology *167*, 400–406.

Mathor, M.B., Ferrari, G., Dellambra, E., Cilli, M., Mavilio, F., Cancedda, R., and De Luca, M. (1996). Clonal analysis of stably transduced human epidermal stem cells in culture. Proc. Natl. Acad. Sci. USA *93*, 10371–10376.

Matsui, C., Pereira, P., Wang, C.K., Nelson, C.F., Kutzkey, T., Lanigan, C., Woodley, D., Morohashi, M., Welsh, E.A., and Hoeffler, W.K. (1998). Extent of laminin-5 assembly and secretion effect junctional epidermolysis bullosa phenotype. J. Exp. Med. *187*, 1273–1283.

Mavilio, F., Pellegrini, G., Ferrari, S., Di Nunzio, F., Di Iorio, E., Recchia, A., Maruggi, G., Ferrari, G., Provasi, E., Bonini, C., et al. (2006). Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. Nat. Med. *12*, 1397–1402.

McMillan, J.R., McGrath, J.A., Pulkkinen, L., Kon, A., Burgeson, R.E., Ortonne, J.P., Meneguzzi, G., Uitto, J., and Eady, R.A. (1997). Immunohistochemical analysis of the skin in junctional epidermolysis bullosa using laminin 5 chain specific antibodies is of limited value in predicting the underlying gene mutation. Br. J. Dermatol. *136*, 817–822.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature *398*, 708–713.

Nguyen, B.P., Gil, S.G., and Carter, W.G. (2000). Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. J. Biol. Chem. *275*, 31896–31907.

Pan, D. (2010). The hippo signaling pathway in development and cancer. Dev. Cell *19*, 491–505.

Panciera, T., Azzolin, L., Fujimura, A., Di Biagio, D., Frasson, C., Bresolin, S., Soligo, S., Basso, G., Bicciato, S., Rosato, A., et al. (2016). Induction of Expandable Tissue-Specific Stem/Progenitor Cells through Transient Expression of YAP/TAZ. Cell Stem Cell *19*, 725–737.

Pellegrini, G., Traverso, C.E., Franzi, A.T., Zingirian, M., Cancedda, R., and De Luca, M. (1997). Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet *349*, 990–993.

Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., Bonini, S., Rama, P., and De Luca, M. (1999a). Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J. Cell Biol. *145*, 769–782.

Pellegrini, G., Ranno, R., Stracuzzi, G., Bondanza, S., Guerra, L., Zambruno, G., Micali, G., and De Luca, M. (1999b). The control of epidermal stem cells (holoclones) in the treatment of massive full-thickness burns with autologous keratinocytes cultured on fibrin. Transplantation *68*, 868–879.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., and De Luca, M. (2001). p63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. USA *98*, 3156–3161.

Pellegrini, G., Rama, P., Matuska, S., Lambiase, A., Bonini, S., Pocobelli, A., Colabelli, R.G., Spadea, L., Fasciani, R., Balestrazzi, E., et al. (2013). Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. Regen. Med. *8*, 553–567.

Piccolo, S., Cordenonsi, M., and Dupont, S. (2013). Molecular pathways: YAP and TAZ take center stage in organ growth and tumorigenesis. Clin. Cancer Res. *19*, 4925–4930.

Rama, P., Matuska, S., Paganoni, G., Spinelli, A., De Luca, M., and Pellegrini, G. (2010). Limbal stem-cell therapy and long-term corneal regeneration. N. Engl. J. Med. *363*, 147–155.

Rheinwald, J.G., and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell *6*, 331–343.

Rochat, A., Kobayashi, K., and Barrandon, Y. (1994). Location of stem cells of human hair follicles by clonal analysis. Cell *76*, 1063–1073.

Rodin, S., Domogatskaya, A., Ström, S., Hansson, E.M., Chien, K.R., Inzunza, J., Hovatta, O., and Tryggvason, K. (2010). Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat. Biotechnol. *28*, 611–615.

Ronfard, V., Rives, J.M., Neveux, Y., Carsin, H., and Barrandon, Y. (2000). Long-term regeneration of human epidermis on third degree burns transplanted with autologous cultured epithelium grown on a fibrin matrix. Transplantation *70*, 1588–1598.

Rousselle, P., and Beck, K. (2013). Laminin 332 processing impacts cellular behavior. Cell Adhes. Migr. *7*, 122–134.

Rousselle, P., Keene, D.R., Ruggiero, F., Champliaud, M.F., Rest, M., and Burgeson, R.E. (1997). Laminin 5 binds the NC-1 domain of type VII collagen. J. Cell Biol. *138*, 719–728.

Ryan, M.C., Lee, K., Miyashita, Y., and Carter, W.G. (1999). Targeted disruption of the LAMA3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. J. Cell Biol. *145*, 1309–1323.

Sambandam, S.A.T., Kasetti, R.B., Xue, L., Dean, D.C., Lu, Q., and Li, Q. (2015). 14-3-3σ regulates keratinocyte proliferation and differentiation by modulating Yap1 cellular localization. J. Invest. Dermatol. *135*, 1621–1628.

Schlegelmilch, K., Mohseni, M., Kirak, O., Pruszak, J., Rodriguez, J.R., Zhou, D., Kreger, B.T., Vasioukhin, V., Avruch, J., Brummelkamp, T.R., and Camargo, F.D. (2011). Yap1 acts downstream of α-catenin to control epidermal proliferation. Cell *144*, 782–795.

Senoo, M. (2013). Epidermal Stem Cells in Homeostasis and Wound Repair of the Skin. Adv. Wound Care (New Rochelle) *2*, 273–282.

Senoo, M., Pinto, F., Crum, C.P., and McKeon, F. (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. Cell *129*, 523–536.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602.

Simpson, C.L., Patel, D.M., and Green, K.J. (2011). Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. Nat. Rev. Mol. Cell Biol. *12*, 565–580.

Siprashvili, Z., Nguyen, N.T., Gorell, E.S., Loutit, K., Khuu, P., Furukawa, L.K., Lorenz, H.P., Leung, T.H., Keene, D.R., Rieger, K.E., et al. (2016). Safety and Wound Outcomes Following Genetically Corrected Autologous Epidermal Grafts in Patients With Recessive Dystrophic Epidermolysis Bullosa. JAMA *316*, 1808–1817.

Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, L.M., Falcioni, R., Kennel, S.J., Aplin, J.D., Baker, J., Loizidou, M., et al. (1991). Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. J. Cell Biol. *113*, 907–917.

Stepp, M.A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I.K. (1990). Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. Proc. Natl. Acad. Sci. USA *87*, 8970–8974.

Takayama, K., Nagamoto, Y., Mimura, N., Tashiro, K., Sakurai, F., Tachibana, M., Hayakawa, T., Kawabata, K., and Mizuguchi, H. (2013). Long-term self-renewal of human ES/iPS-derived hepatoblast-like cells on human laminin 111-coated dishes. Stem Cell Reports *1*, 322–335.

Walko, G., Castañón, M.J., and Wiche, G. (2015). Molecular architecture and function of the hemidesmosome. Cell Tissue Res. *360*, 363–378.

Walko, G., Woodhouse, S., Pisco, A.O., Rognoni, E., Liakath-Ali, K., Lichtenberger, B.M., Mishra, A., Telerman, S.B., Viswanathan, P., Logtenberg, M., et al. (2017). A genome-wide screen identifies YAP/WBP2 interplay conferring growth advantage on human epidermal stem cells. Nat. Commun. *8*, 14744.

Watt, F.M. (2002). Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J. *21*, 3919–3926.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dötsch, V., Andrews, N.C., Caput, D., and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol. Cell *2*, 305–316.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature *398*, 714–718.

Zhang, H., Pasolli, H.A., and Fuchs, E. (2011). Yes-associated protein (YAP) transcriptional coactivator functions in balancing growth and differentiation in skin. Proc. Natl. Acad. Sci. USA *108*, 2270–2275.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. *21*, 2747–2761.

Zhao, R., Fallon, T.R., Saladi, S.V., Pardo-Saganta, A., Villoria, J., Mou, H., Vinarsky, V., Gonzalez-Celeiro, M., Nunna, N., Hariri, L.P., et al. (2014). Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. Dev. Cell *30*, 151–165.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Laminin β-3 (6F12)	Patricia Rousselle Laboratory, Lyon	N/A
P63α	Di Iorio et al., 2005	N/A
YAP1	Merck	Cat# MABC203; RRID: AB_11203473
Phospho-YAP (Ser127)	Cell Signaling	Cat# 4911; RRID: AB_2218913
YAP	Cell Signaling	Cat# 4912; RRID: AB_2218911
Survivin	Abcam	Cat# ab469; RRID: AB_304564
TAZ	BD PharMingen	Cat# 560235; RRID: AB_1645338
P16 (C-20)	Santa Cruz	Cat# sc-468; RRID: AB_632103
P53 (DO-1)	Santa Cruz	Cat# sc-126; RRID: AB_628082
Laminin β-3 (C-19)	Santa Cruz	Cat# sc-7651; RRID: AB_2134210
Integrin beta 1	Abcam	Cat# ab52971; RRID: AB_870695
Integrin beta 4	Santa Cruz	Cat# sc-135950; RRID: N/A
COL7A1 (LH7.2)	Santa Cruz	Cat# sc-53226; RRID: AB_1121804
14-3-3 sigma (1.N.6)	Abcam	Cat# ab14123; RRID: AB_300927
alpha 1 Catenin (EP1793Y)	Abcam	Cat# ab51032; RRID: AB_868700
donkey anti-mouse IgG-HRP	Santa Cruz	Cat# sc-2314; RRID: AB_641170
donkey anti-rabbit IgG-HRP	Santa Cruz	Cat# sc-2313; RRID: AB_641181
Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	Cat# A10037; RRID: AB_2534013
Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-21206; RRID: AB_2535792
Bacterial and Virus Strains		
5-alpha Competent *E. coli* (High Efficiency)	New England BioLabs	Cat# C2987H
Biological Samples		
Human healthy skin donors for primary keratinocytes and skin sections	This manuscript	N/A
Skin from Junctional Epidermolysis Bullosa Patients for primary keratinocytes and skin sections	This manuscript	N/A
Skin from Dystrophic Epidermolysis Bullosa Patients for primary keratinocytes and skin sections	This manuscript	N/A
Chemicals, Peptides, and Recombinant Proteins		
DMEM High Glucose	EuroClone	Cat# ECB7501L
Ham's F-12	Corning	Cat# 10-080-CVR
Penicillin-Streptomycin Solution 100X	EuroClone	Cat# ECB3001D
L-Glutamine 100X, 200mM	EuroClone	Cat# ECB3000D
Humulin R 100 UI/ml	Lilly	Cat# HI0210
Adenine	Merck	Cat# 1152 CAS: 73-24-5
Epidermal Growth Factor H. Recombinant	Austral Biologicals	Cat# GF-010-8
QD Cholera Toxin	List Biological	Cat# 9100B
Hydrocortisone	Acros	Cat# AC352450010 CAS: 50-23-7
Liothyronine Sodium (triiodothyronine)	Peptido	N/A
Trypsin-EDTA (0.5%)	Life Technologies	Cat# 15400054
Fetal Bovine Serum Gamma Irradiated	Life Technologies	Cat# 10099

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donor Adult Bovine Serum Gamma Irradiated	HyClone	Cat# SH30075.03
Bovine Serum Albumin (BSA)	Sigma Aldrich	Cat# A2153 CAS: 9048-46-8
DAPI	Roche	Cat# 10236276001 CAS: 28718-90-3
Cryostat embedding medium killik	Bio-Optica	Cat# 05-9801
Fluorescence Mounting Medium	Agilent Dako	Cat# S3023
Triton X-100 Detergent	Bio Rad	Cat# 1610407
Tween 20	Bio Rad	Cat# 1706531
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	Cat# 34580
RIPA Buffer	Sigma Aldrich	Cat# R0278
Nitrocellulose Membrane, 0.45 μm	Bio Rad	Cat# 1620115
NuPAGE 4-12% Bis-Tris Protein Gels, 1.5 mm, 10-well	Thermo Fisher Scientific	Cat# NP0335BOX
Halt Phosphatase Inhibitor Cocktail (100X)	Thermo Fisher Scientific	Cat# 78420
Halt Protease Inhibitor Cocktail (100X)	Thermo Fisher Scientific	Cat# 87786
Paraformaldehyde	Sigma Aldrich	Cat# P6148 CAS: 30525-89-4
TRIzol RNA Isolation Reagents	Thermo Fisher Scientific	Cat# 15596026
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific	Cat# 4304437
Lipofectamine RNAiMAX Transfection Reagent	Thermo Fisher Scientific	Cat# 13778030
Biolaminin 332 LN (LN332)	BioLamina	Cat# LN332-0202
Rhodamine B	Sigma Aldrich	Cat# R6626
EDTA	Carlo Erba	Cat# 303227 CAS: 6381-92-6
Formaldehyde solution	Sigma Aldrich	Cat# F1635 CAS: 50-00-0
Glutaraldehyde solution	Sigma Aldrich	Cat# G5882 CAS: 111-30-8
X-gal	Sigma Aldrich	Cat# B4252 CAS: 7240-90-6
Polybrene	Sigma Aldrich	Cat# TR-1003-G
Critical Commercial Assays		
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23227
NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit	Thermo Fisher Scientific	Cat# 78833
SuperScript VILO cDNA Synthesis Kit	Thermo Fisher Scientific	Cat# 11754050
PureLink RNA Mini Kit	Thermo Fisher Scientific	Cat# 12183018A
Experimental Models: Cell Lines		
Am12-MGFLAMB3 producer cell lines	Molmed S.p.a.	N/A
HEK293T	ATCC	Cat# CRL-3216; RRID: CVCL_0063
Oligonucleotides		
Stealth RNAi siRNA Negative Control Hi GC	Thermo Fisher Scientific	Cat# 12935400
Stealth RNAi siRNA Negative Control Med GC Duplex #2	Thermo Fisher Scientific	Cat# 12935112
Stealth RNAi siRNA YAP GGCAAAGACAUCUUCUGGUCAGAGA	Thermo Fisher Scientific	N/A
Stealth RNAi siRNA YAP CAUCUUCUGGUCAGAGAUACUUCUU	Thermo Fisher Scientific	N/A
Stealth RNAi siRNA TAZ AAAGUUCCUAAGUCAACGUUUGUUC	Thermo Fisher Scientific	N/A
Stealth RNAi siRNA TAZ CUGAGACAUGGCUUCACUCACGUCA	Thermo Fisher Scientific	N/A
Silencer Negative Control No. 1 siRNA	Thermo Fisher Scientific	Cat# AM4611
Silencer Negative Control No. 2 siRNA	Thermo Fisher Scientific	Cat# AM4613
Silencer® Select Human LAMB3 (s8074, s8075, s8076)	Thermo Fisher Scientific	Cat# 4392421
Silencer® Select Human YAP1 (s20368, s20366)	Thermo Fisher Scientific	Cat# 4392421
Silencer® Select Human WWTR1 (s24788, s24787)	Thermo Fisher Scientific	Cat# 4392421
Silencer® Select Human ITGA6 (s7493, s7494)	Thermo Fisher Scientific	Cat# 4390824
Silencer® Select Human ITGB4 (s7585)	Thermo Fisher Scientific	Cat# 4392420
Silencer® Select Human ITGB1 (s7574, s7575)	Thermo Fisher Scientific	Cat# 4390824
TaqMan® probe YAP1 Hs00902712_g1	Thermo Fisher Scientific	Cat# 4331182

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TaqMan® probe CYR61 Hs00998500_g1	Thermo Fisher Scientific	Cat# 4331182
TaqMan® probe CTGF Hs01026927_g1	Thermo Fisher Scientific	Cat# 4331182
TaqMan® probe BIRC5 Hs03043576_m1	Thermo Fisher Scientific	Cat# 4331182
TaqMan® probe ITGA6 Hs01041011_m1	Thermo Fisher Scientific	Cat# 4331182
TaqMan® probe ITGB1 Hs00559595_m1	Thermo Fisher Scientific	Cat# 4331182
Recombinant DNA		
GIPZ non-silencing Lentiviral shRNA Control	Dharmacon	Cat# RHS4346
GIPZ Human WWTR1 shRNA #1	Dharmacon	Cat# RHS4430-200267049
GIPZ Human WWTR1 shRNA #2	Dharmacon	Cat# RHS4430-200268588
TRIPZ Inducible non-silencing Lentiviral shRNA Control	Dharmacon	Cat# RHS4743
TRIPZ Human YAP1 shRNA #1	Dharmacon	Cat# RHS4696-200686207
TRIPZ Human YAP1 shRNA #2	Dharmacon	Cat# RHS4696-200707120
pCW57.1-eGFP	Laboratory of prof. S. Piccolo	N/A
pCW57.1-Flag.YAP1	Laboratory of prof. S. Piccolo	N/A
pCW57.1-Flag.YAP1S127A	Laboratory of prof. S. Piccolo	N/A
pCW57.1-Flag.YAP5SA	Laboratory of prof. S. Piccolo	N/A
pCW57.1-Flag.YAPS94A	Laboratory of prof. S. Piccolo	N/A
Software		
GraphPad Prism 7	GraphPad	https://www.graphpad.com
Fiji	ImageJ	https://fiji.sc
Zen 2009 (confocal microscope LSM510meta)	Zeiss	https://www.zeiss.de
AxioVision Rel. 4.8 (Microscope AXIO ImagerA1)	Zeiss	https://www.zeiss.de

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Michele De Luca (michele.deluca@unimore.it).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human tissues

All human tissues were collected after informed consent and in compliance with Italian regulations (Comitato Etico Provinciale, C.E. N. 178/09 and C.E. N. 124/2016), German (District Council of Arnsberg, North Rhine-Westphalia, Germany), and Austrian (Austrian Ministry of Health) regulations.

Primary human cell cultures

Human skin samples were obtained as anonymised surgical waste, typically from abdominoplasty or mammoplasty. Briefly, skin biopsies were minced and trypsinized (0.05% trypsin/0.01%EDTA) at 37°C for 4 hr. Cells were collected every 30 min, plated ($2.5 \times 10^4/cm^2$) on lethally irradiated 3T3-J2 cells ($2.4 \times 10^4/cm^2$) and cultured in 5% CO2 and humidified atmosphere in keratinocyte growth medium (KGM): DMEM and and Ham's F12 media (3:1 mixture) containing Fetal Bovine Serum (FBS) (10%), insulin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (CT, 0.1 nM), triiodothyronine (Liothyronine Sodium) (2 nM), epidermal growth factor (EGF, 10 ng/ml), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml) and cultivated on a feeder-layer of lethally irradiated 3T3-J2 cells as described (Dellambra et al., 1998) . Subconfluent primary cultures were serially propagated as described (De Luca et al., 1988; Mathor et al., 1996). For serial propagation, cells were passaged at the stage of subconfluence, until they reached senescence (Pellegrini et al., 1999b).

Primary Junctional Epidermolysis Bullosa (JEB) and Dystrophic Epidermolysis Bullosa (RDEB) cell cultures

Primary EB keratinocytes were cultivated as described above from a 1-4cm^2 biopsies taken (after informed consent) from 4 patients suffering from LAMB3-dependent Junctional Epidermolysis Bullosa (JEB 2-5) and 2 patients suffering from Col7A1-dependent Dystrophic Epidermolysis Bullosa (RDEB 1-2), ranging in age from 3 months to 49 years(Bauer et al. 2017; Hirsch et al., 2017; Mavilio et al., 2006) .

3T3J2 cell line

Mouse 3T3-J2 cells were a gift from Prof. Howard Green, Harvard Medical School (Boston, MA, USA). A clinical grade 3T3-J2 cell bank was established under GMP standards by a qualified contractor (EUFETS, GmbH, Idar-Oberstein, Germany), according to the ICH guidelines. GMP-certified 3T3-J2 cells have been authorized for clinical use by national and European regulatory authorities and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% irradiated donor adult bovine serum gamma irradiated, glutamine (4 mM) and penicillin-streptomycin (50 IU/ml).

MFG-LAMB3-Packaging cell line

A retroviral vector expressing the full-length 3.6-kb *LAMB3* cDNA under the control of the MLV LTR was constructed by cloning a 3.6-kb of LAMB3 cDNA (Gene Bank Accession #Q13751) into MFG-backbone (Markowitz et al., 1988). A 5′ fragment of LAMB3 cDNA (563bp) from the ATG to StuI site was obtained by PCR using as template the LB3SN plasmid (Dellambra et al., 1998). The PCR product was cloned into NcoI and BamHI sites of MFG-vector. The second fragment of LAMB3 cDNA (3050bp) was obtained from LB3SN by enzyme digestion from StuI to XmnI and cloned into MGF-vector into StuI site. The entire cDNA of LAMB3 was fully sequenced. The Am12-MGFLAMB3 producer cell lines (Mathor et al., 1996) were generated by transinfection in the amphotropic Gp+*env*Am12 packaging cell line (Mathor et al., 1996). Briefly, plasmid DNA was introduced into the GP+E86 ecotropic packaging cell line by standard calcium phosphate transfection. Forty-eighth ours after transfection, supernatant was harvested and used to infect the amphotropic packaging cell line GP+envAml2 ATCC n° CRL 9641(Markowitz et al., 1988) for 16h in the presence of 8 ug/ml Polybrene. Infected Am12 cells were clonally selected in HXM medium supplemented with 10% FCS and containing 0.8mg/ml G418 and 0.2mg/ml hygromycin B (Sigma). Single colonies were screened for human LAMB3 production by immunofluorescence using an antibody specific for LAMB3 6F12 monoclonal antibody (from Dr. Patricia Rousselle, CNRS, Lyon) and for viral titer. The resulting producer cell lines showed a viral titer of $2X10^6$ colony-forming units (cfu). A master cell bank of a high-titer packaging clone (Am12-*LAMB3* 2/8) was made under GMP standards by a qualified contractor (Molmed S.p.A, Milan, Italy) according to the ICH guidelines and cultured in DMEM supplemented with 10% irradiated fetal bovine serum, glutamine (2 mM), and penicillin-streptomycin (50 IU/ml). All certifications, quality and safety tests (including detection on viruses and other micro-organisms both *in vitro* and *in vivo*) were performed under GMP standards for both cell lines.

METHOD DETAILS

Generation of genetically corrected epidermal keratinocytes

In brief, sub-confluent primary cultures derived from JEB skin biopsies were trypsinized (0.05% trypsin and 0.01% EDTA) at 37 °C for 15–20 min and seeded (1.33 × 104 cells per cm2) onto a feeder layer (8 × 104 cells per cm2) composed of lethally irradiated 3T3-J2 cells and producer GP+envAm12-LAMB3 cells12 (a 1:2 mixture) in KGM. After 3 days of cultivation, cells were collected and cultured in KGM on a regular 3T3-J2 feeder layer. At each step, efficiency of colony formation by keratinocytes was determined by plating 1,000 cells and staining them 12 days later with rhodamine B.

Colony Forming Efficiency, population doublings and growth rate

Colony forming efficiency of keratinocytes at each culture amplification was determined by plating 1000 cells on parallel 100-mm dishes and staining them 12 days later with rhodamine B. Total colonies were calculated as a percentage of total plated cells; aborted colonies (made by large and flattened, terminally differentiated cells) were calculated as a percentage of total colonies. The number of cell generations performed by cultured keratinocytes was then calculated using the following formula: x = 3.322 logN/No, where N is the total number of cells obtained at each passage and No is the number of clonogenic cells plated (Rochat et al., 1994). CFE was determined during serial cultivation at each passage. Growth rates were calculated using the following formula log ((number of cells obtained)/(number of cells seeded))/log(2) (Bisson et al., 2013).

Clonal analysis

Sub-confluent epidermal cultures were trypsinized, serially diluted and plated in 96-well plates (0.5-1 cells per well). After 7 days of cultivation, single clones were identified under an inverted microscope and trypsinized. One-quarter of the clone was cultured for 12 days onto a 100-mm (indicator) dish, which was then fixed and stained with rhodamine B for the classification of clonal type 12-d after (Figure S1). The remaining three-quarters of the clone was cultivated on 6-well plates for 4–5 days and then used to prepare cell extracts for further analyses.

Western blotting

All samples destined to western blot analysis were depleted of feeder layer by gently removal of feeder layer in cold PBS/EDTA 20mM and then keratinocytes colonies were collected by scraping. Protein samples from whole cell (1 × RIPA buffer, Sigma Aldrich), nucleus/cytoplasmic (NE-PER, Thermo Fisher) were supplemented with Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Total protein amount was quantified in RIPA extracts using the BCA kit (Pierce). Equivalent quantities of RIPA-solubilized proteins were resolved by SDS-PAGE in 4%–12% NuPAGE Bis-Tris Gels (ThermoFisher) and transferred 100V at 4°C for 2 hours onto nitrocellulose membrane (Millipore). Membranes were blocked with either 5% (w/v) non-fat milk or 5% (w/v) Bovine Serum

Albumin (BSA) in PBS supplemented with 0.05% (v/v) Tween-20 (PBS-T) and then probed with the indicated antibodies diluted in blocking buffer. Primary antibody-probed blots were visualized with appropriate horseradish peroxidase-coupled secondary antibodies (Santa Cruz). Protein detection was carried out using a chemiluminescent substrate (Thermo Fisher).

The following antibodies were used for western blot: p63α rabbit purified anti-p63α immunoglobulin G (IgG; PRIMM) (Di Iorio et al., 2005), Yap (Millipore), Phospho-Yap Ser 127 (Cell Signaling), Survivin (Abcam), Taz (BD PharMingen), Laminin332-β3, p16 (Santa Cruz), p53 (Santa Cruz), Integrin β1 (Abcam), Integrin β4 (Santa Cruz).

Immunofluorescence

The following antibodies were used for immunofluorescence: mouse 6F12 monoclonal antibody to laminin 332-β3 (a gift from Patricia Rousselle, IBCP), Col7A1 monoclonal antibody (Santa Cruz), rabbit purified anti-p63α immunoglobulin G (IgG; PRIMM) (Di Iorio et al., 2005), 14-3-3-σ (Abcam), YAP1 (Millipore), Phospho-Yap Ser 127 (Cell Signaling), YAP (Cell Signaling), alpha-Catenin (Abcam).

For immunofluorescence analysis cells were seeded at 1.000/wells onto glass coverslips and grown in the presence of feeder cells in keratinocyte medium for 4, 6 or 10 days and then fixed with PFA 3% (10 min, at RT). The coverslips were carefully washed with phosphate-buffered saline (PBS), permeabilized with PBS/triton 0.5% for 15 min and then blocked for 1 h at room temperature with FBS 5%/2% BSA in PBS/triton 0.5%. Primary antibodies for immunofluorescence were incubated overnight in FBS 5%/2% BSA in PBS/triton 0.5%. Alexa Fluor 568 donkey anti-mouse (Thermo Fisher), Alexa Fluor 488 donkey anti-rabbit (Thermo Fisher), diluted 1:2.000 in 5% Goat serum, 2% BSA in PBS/triton 0.5% for 1h at RT. Cell nuclei were stained with DAPI. Glasses were then mounted with Dako Mounting medium and fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar 40 × /1.3 oil immersion objective or Zeiss Microscope AXIO ImagerA1 with EC-Plan Neofluar 20 × /0.5 objective or 10 × /0.5 objective.

For *in vivo* immunofluorescence, skin biopsies derived from healthy donors or JEB 1, JEB2 and JEB3 were washed in PBS, embedded in Killik-OCT cryostat embedding medium (Bio-Optica) and frozen. Immunofluorescence was performed on 7-μm skin sections (fixed in PFA 3%, permeabilized with PBS/triton 0.2% for 15 min at room temperature and blocked for 1 h at room temperature with BSA 2% in PBS/triton 0.2%) using the previous described antibodies in BSA 2% in PBS/triton 0.2% and added to skin sections for 30 min at 37 °C. Sections were washed three times in PBS/triton 0.1% and incubated with Alexa Fluor 568 donkey anti-mouse (Thermo Fisher), Alexa Fluor 488 donkey anti-rabbit (Thermo Fisher), diluted 1:2.000 in BSA 2%, PBS/triton 0.2% for 30 min at 37 °C. Cell nuclei were stained with DAPI. Glasses were then mounted with Dako Mounting medium and fluorescent signals were monitored under a Zeiss confocal microscope LSM510meta with a Zeiss EC Plan-Neofluar 40 × /1.3 oil immersion objective.

Senescence-Associated β-Galactosidase Staining

Cells were washed in PBS, fixed for 3-5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed, and incubated with staining solution at 37°C (no CO_2) from 2-4 hours to 12-16 hours. Staining solution contain: 40 mM citric acid/Na phosphate buffer, 5 mM $K_4[Fe(CN)_6]$ $3H_2O$, 5 mM $K_3[Fe(CN)_6]$, 150 mM sodium chloride, 2 mM magnesium chloride and 1 mg ml^{-1} X-gal in distilled water (Debacq-Chainiaux et al., 2009).

Plasmid constructs

For inducible expression of YAP constructs, cDNA for human HA-tagged -YAP1, YAP1S127A, YAP15SA (LATS mutant sites) and human YAP1S94A (TEAD-binding mutant) (Panciera et al., 2016) (gifted from Stefano Piccolo) were subcloned NheI/SalI into pCW57.1. HA-tag-eGFP was derived from p-babe pure, cloned into NheI blut/SalI into pCW57.1 and used as control. All constructs were confirmed by sequencing. shRNA constructs were purchased from Dharmacon (Table S1).

Lentiviral production and primary human keratinocyte infection

HEK293T cells were kept in DMEM supplemented with 10% FBS, 1% Glutamine and 1% Pen/Strep antibiotics (Thermo Fisher). Lentiviral particles were prepared by transiently transfecting HEK293T with lentiviral vectors together with packaging vectors pMD2-VSVG and pPAX2 by using calcium phosphate transfection. Infectious lentiviruses were harvested after 48h post-transfection and filtered through 0.45- μm-pore cellulose acetate filters. Recombinant lentiviruses were concentrated by ultracentrifugation. Primary human keratinocytes were infected with the lentiviral particles at MOI10 in the presence of 8 μg/mL polybrene (Sigma). The cells were harvested 5-6 days after transduction for further analyses.

Transient transfection

A total amount of 100nM siRNA (Stealth siRNA, Invitrogen) for YAP, TAZ, control medium GC rich and high GC rich siRNA (Stealth siRNA, Invitrogen) (Table S2) were transfected by Lipofectamine RNAiMAX (Thermo Fisher). for 5h in the absence of serum. After 5h the medium was changed and replacing in Kc medium. For LAMB3 and p63 a total amount of 100nM of specific siRNA (Silencer, Invitrogen) was transfected (Table S3). The cells were collected 48h after transfection in RIPA buffer or in TriZol (Thermo Fisher).

RNA extraction and real-time qPCR

Total RNA was isolated from cultured cells using the PureLink RNA Mini Kit (Thermo Fisher). Complementary DNA was generated using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher). Real-time qPCR analyses were carried out on triplicate samplings

of retrotranscribed cDNAs with 7500 Real-Time PCR System (Thermo Fisher). Expression levels are given relative to GAPDH. List of TaqMan probe (Thermo Fisher) are provided in Table S4.

Coating of Laminin 332

To culture JEB keratinocytes onto laminin 332-coated dishes, six well plates were coated with human recombinant laminin-332 (BioLamina, https://www.biolamina.com) diluted in DPBS at final concentration 1mg/cm^2 and incubated overnight at +4°C.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

GraphPad Prism version 7 was used for statistical analyzes included in the main and supplementary figures of the study. The statistical details of the experiments including statistical tests used, value of n, and what n represents, can be found in the figure legends. A *p value < 0.05, **p value < 0.01, ***p value < 0.001, and ****p value < 0.0001 were considered statistically significant. RT-qPCR data are presented as the mean ± SEM of at least three independent experiments and the value of n can be found in the figure legends. No statistical methods were used to predetermine sample sizes.

Replications

All experiments were replicated a number of times depending of the experiment performed. For qualitative data such as those obtained from immunofluorescence, pictures are representative of what was observed in at least three independent samples or replicates.