

Epidermal YAP2-5SA- Δ C Drives β -catenin Activation to Promote Keratinocyte Proliferation in Mouse Skin In Vivo

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The epidermis is a highly regenerative tissue. YAP is a pivotal regulator of stem/progenitor cells in tissue regeneration, including in the epidermis. The molecular mechanisms downstream of YAP that activate epidermal cell proliferation remain largely unknown. We found that YAP and β -catenin co-localize in the nuclei of keratinocytes in the regenerating epidermis in vivo and in proliferating HaCaT keratinocytes in vitro. Inactivation of YAP in HaCaT keratinocytes resulted in reduced activated β -catenin and reduced keratinocyte numbers in vitro. In addition, we found that in the hyperplastic epidermis of YAP2-5SA- Δ C mice, the mutant YAP2-5SA- Δ C protein was predominantly localized in the keratinocyte nuclei and caused increased expression of activated nuclear β -catenin. Accordingly, β -catenin transcriptional activity was elevated in the skin of live YAP2-5SA- Δ C/TOPFLASH mice. Lastly, loss of β -catenin in basal keratinocytes of YAP2-5SA- Δ C/K14-creERT/*Cttnb1*^{-/-} mice resulted in reduced proliferation of basal keratinocytes and a striking rescue of the hyperplastic abnormalities. Taken together, our work shows that YAP2-5SA- Δ C drives β -catenin activity to promote basal keratinocyte proliferation in the mouse skin in vivo. Our data shine new light on the etiology of regenerative dermatological disorders and other human diseases that display increased YAP and β -catenin activity.

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INTRODUCTION

The remarkable regenerative capacity of the epidermis is enabled by stem/progenitor cell populations, which are key to normal skin homeostasis and injury repair but also the development of cancers. How epidermal regeneration is regulated molecularly remains one of the exciting questions in skin biology research.

YAP and its paralog, TAZ, are expressed in stem/progenitor cell compartments of vertebrate tissues, where they control cell proliferation, tissue homeostasis, and organ size (Camargo et al., 2007; Dong et al., 2007). They act as transcriptional co-activators that are classically known to be downstream effectors of the highly conserved Hippo kinase pathway. In the unphosphorylated state they will translocate to the nucleus and bind the TEAD transcription factors to activate target gene transcription and cell proliferation (reviewed in Piccolo et al., 2014; Yu et al., 2015). Recent

pioneering work showed that local mechanical cues play a major overarching role and can override the activity of the core Hippo kinases in controlling YAP activity (Aragona et al., 2013; Dupont et al., 2011).

In the skin, YAP is expressed throughout the epidermis, including in the basal epidermal stem/progenitor cell populations (Beverdam et al., 2013; Zhang et al., 2011). Overexpression of YAP protein mutants in basal keratinocytes results in increased proliferation rates and causes epidermal hyperplasia, whereas YAP loss-of-function in the basal epidermis resulted in epidermal hypoplasia (Beverdam et al., 2013; Schlegelmilch et al., 2011; Zhang et al., 2011). These studies show that YAP plays a pivotal role in controlling epidermal homeostasis. However, the downstream mechanisms regulated by YAP in the control of basal epidermal cell proliferation remain unknown.

The Wnt/ β -catenin pathway plays a pivotal role in epidermal development and regeneration (Huelsen et al., 2001; Lo Celso et al., 2004; Lowry et al., 2005; Van Mater et al., 2003; Watt and Collins, 2008). Wnt pathway activation results in the dissociation of the cytosolic β -catenin destruction complex and the nuclear translocation of β -catenin, where it activates gene transcription and cell proliferation (Clevers et al., 2014). There is increasing evidence that the Hippo/YAP and Wnt/ β -catenin signaling pathways interact to control cell proliferation and tissue homeostasis (e.g., reviewed in Piccolo et al., 2014; Yu et al., 2015). However, it remains unclear if this is also the case in the control of stem/progenitor cells during epidermal homeostasis.

In this study we show that YAP and β -catenin are expressed in strongly overlapping patterns within the epidermis of mice

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; K, keratin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P, postnatal day; qRT-PCR, quantitative real-time reverse transcriptase-PCR; siRNA, small interfering RNA

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and provide experimental support for the hypothesis that YAP and β -catenin cooperate in the control of HaCaT keratinocyte proliferation *in vitro*. Using a viable transgenic mouse line in which the YAP2-5SA- Δ C mutant is predominantly expressed in basal keratinocytes, we found that YAP2-5SA- Δ C drives β -catenin activation to promote basal keratinocyte proliferation in the mouse skin *in vivo*. Together, our findings indicate that YAP positively regulates β -catenin activity in the control of normal epidermal homeostasis, which may be perturbed in regenerative skin disease such as skin cancer displaying increased YAP activity.

RESULTS

YAP and β -catenin co-localize in the regenerating epidermis *in vivo*

A rapidly increasing number of studies shows crosstalk between the Hippo/YAP and Wnt/ β -catenin pathways in the regulation of cell proliferation, tissue growth, and homeostasis (e.g., reviewed in Piccolo et al., 2014; Yu et al., 2015). Therefore, it is conceivable that YAP and β -catenin also cooperate in the control of epidermal cell proliferation. To address this question, we investigated the expression patterns of YAP and β -catenin in sections of dorsal skin of wild-type mice at the rest phase (telogen) and at the growth phase (anagen) of hair follicle cycling using immunofluorescence analysis. YAP and β -catenin were expressed in strongly overlapping patterns throughout the epidermis both at telogen and anagen (Figure 1a–c and f–h), as previously reported (Beverdam et al., 2013; Lo Celso et al., 2004; Zhang et al., 2011). We also detected nuclei positive for both YAP and β -catenin in anagen skin (arrow heads in Figure 1f–h). These findings are consistent with the hypothesis that YAP and β -catenin cooperate to activate basal keratinocyte proliferation during normal epidermal homeostasis.

YAP-induced proliferation of HaCaT keratinocytes grown *in vitro* is associated with nuclear and activated β -catenin

Next, YAP and β -catenin expression were investigated by immunofluorescence analysis in sparse (proliferating) and dense (quiescent) cultures of HaCaT keratinocytes (Figure 2a–d). High YAP signal was detected predominantly in the Ki67-positive nuclei of proliferating keratinocytes, in line with a previous report (Zhao et al., 2007), where it co-localized with β -catenin (Figure 2a and c). YAP was predominantly localized in the cytosol of quiescent HaCaT keratinocytes (Figure 2b and d), in line with a previous report (Zhao et al., 2007), where it co-localized with β -catenin (Figure 2d). These data show that YAP and β -catenin undergo highly similar nucleocytoplasmic trafficking patterns in HaCaT keratinocytes grown *in vitro*, analogous to those observed in MDCK epithelial cells undergoing strain-induced proliferation (Benham-Pyle et al., 2015). Next, we inactivated YAP in sparse, proliferating HaCaT keratinocytes in small interfering RNA (siRNA) knockdown assays and confirmed a strong reduction of YAP protein expression in YAP siRNA-treated versus scramble RNA-treated HaCaT keratinocytes (Figure 2e, f, and top lane in g). We also observed reduced nuclear activated β -catenin (Figure 2e and f), significantly reduced activated β -catenin versus total β -catenin (Figure 2g and h) ($P < 0.05$, $n = 3$), and reduced HaCaT keratinocyte numbers as determined in MTT assays (Figure 2i) ($P < 0.05$,

$n = 3$) in YAP siRNA-treated versus scramble RNA-treated HaCaT keratinocytes. These data show that YAP-induced proliferation of HaCaT keratinocytes grown *in vitro* is associated with nuclear and activated β -catenin, consistent with the hypothesis that YAP and β -catenin cooperate in the control of keratinocyte proliferation.

Increased nuclear and activated β -catenin in keratinocytes of the hyperplastic YAP2-5SA- Δ C epidermis

To investigate if YAP and β -catenin cooperate in the regulation of basal keratinocyte proliferation in the skin *in vivo*, the effect of YAP2-5SA- Δ C expression in the basal epidermis on β -catenin was investigated in skin of YAP2-5SA- Δ C transgenic mice (Beverdam et al., 2013). We detected the myc-tagged YAP2-5SA- Δ C protein predominantly in the nuclei of basal keratinocytes of YAP2-5SA- Δ C skin (Figure 3e). Moreover, we found that YAP2-5SA- Δ C expression in the basal epidermis resulted in increased keratinocyte proliferation, a 3- to 4-fold increase in keratinocyte numbers (Figure 3c), eventually causing epidermal hyperplasia (Figure 3a and b), as we previously reported (Beverdam et al., 2013). This phenotype is similar to that observed in YAPS127A transgenic mouse lines that inducibly express constitutively active YAP (Schlegelmilch et al., 2011; Zhang et al., 2011). Therefore, YAP2-5SA- Δ C expression in the basal epidermis of mouse skin *in vivo* promotes keratinocyte proliferation.

Immunofluorescence analysis was performed on dorsal skin of P85 wild-type and YAP2-5SA- Δ C littermates. Relatively high YAP expression levels were detected throughout the hyperplastic epidermis and hair follicles of YAP2-5SA- Δ C littermates, with an increased percentage of YAP-positive nuclei versus total nuclei compared with wild-type skin ($P < 0.05$, $n = 3$) (Figure 3h and i). This increased nuclear YAP signal represents YAP2-5SA- Δ C (Figure 3e), further indicating that this mutant YAP protein is mostly localized in keratinocyte nuclei of the YAP2-5SA- Δ C epidermis.

β -catenin detected using an anti-pan- β -catenin antibody mostly localized in cell junctions of keratinocytes populating the interfollicular epidermis and hair follicles of wild-type mice (Figure 3j) and YAP2-5SA- Δ C littermate mice (Figure 3k). We also used the anti-activated β -catenin (unphosphorylated at Ser37) antibody (van Noort et al., 2002) and detected a large number of activated β -catenin-positive nuclei throughout the hyperplastic YAP2-5SA- Δ C (arrowheads in Figure 3m). The percentage of activated β -catenin positive nuclei versus total nuclei was significantly higher both in the interfollicular epidermis ($P < 0.05$, $n = 3$) and in the hair follicles ($P < 0.001$, $n = 3$) of YAP2-5SA- Δ C versus wild-type epidermis (Figure 3n & o). This shows that the increased nuclear accumulation of activated β -catenin cannot be attributed merely to higher keratinocyte numbers in YAP2-5SA- Δ C skin but occurs because of YAP2-5SA- Δ C presence. These data suggest that YAP2-5SA- Δ C promotes β -catenin activity in the hyperplastic epidermis of YAP2-5SA- Δ C mice *in vivo*.

Increased β -catenin transcriptional activity in the skin of YAP2-5SA- Δ C/TOPFLASH transgenic mice during development of the epidermal hyperplasia

To confirm β -catenin activation in the YAP2-5SA- Δ C epidermis, the expression levels of β -catenin direct-target

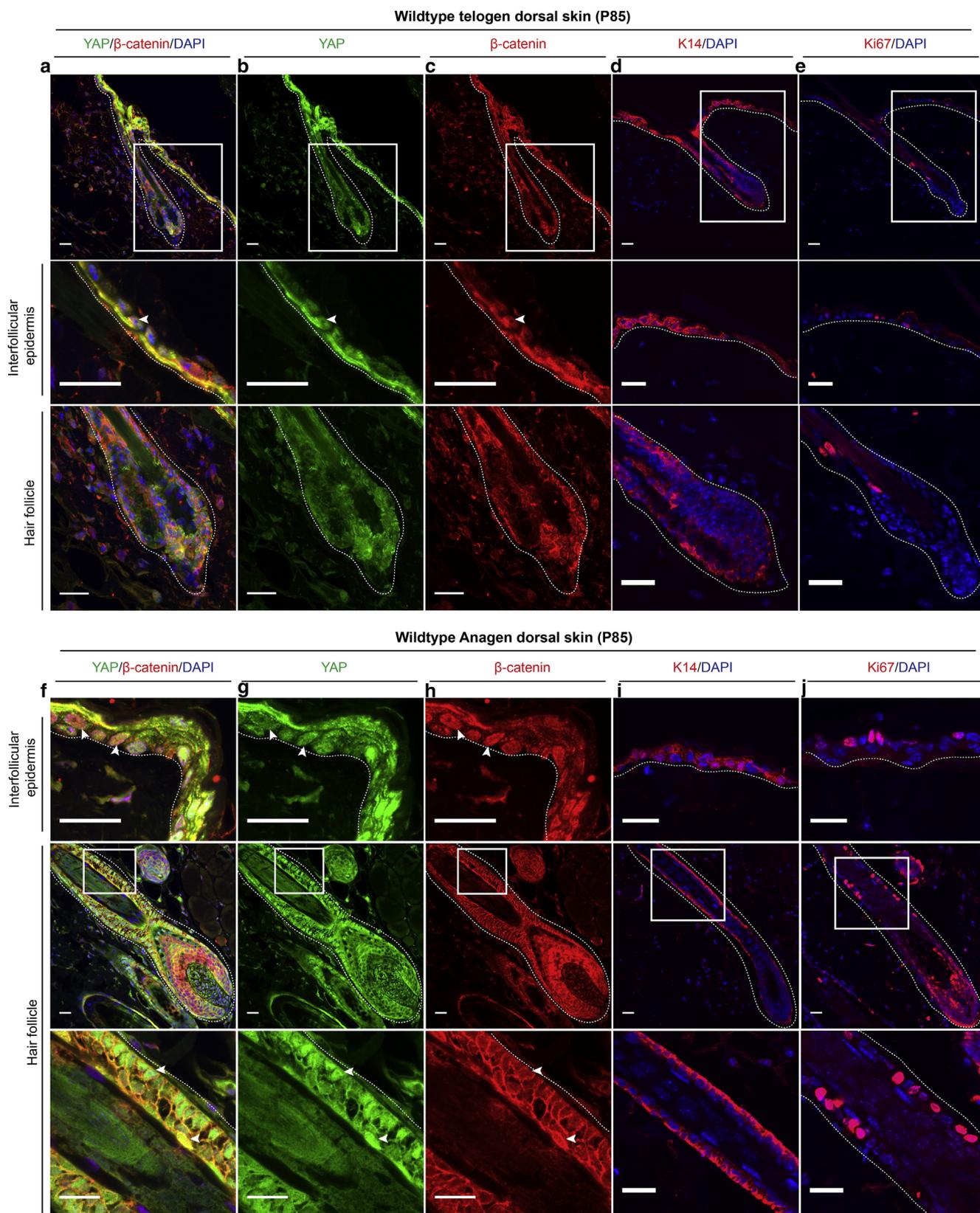


Figure 1. YAP and β -catenin co-localize in the regenerating epidermis in vivo. Immunofluorescence staining of dorsal skin sections at (a–e) telogen and (f–j) anagen of adult wild-type mice detecting (a, b, f, g) YAP, (a, c, f, h) pan β -catenin, (d, i) keratin 14, and (e, j) Ki67. Arrowheads point to (a, b, f, g) YAP- and (a, c, f, h) β -catenin–positive nuclei. Basement membranes and hair follicles are demarcated with dashed lines. P, postnatal day. Scale bars = 20 μ m.

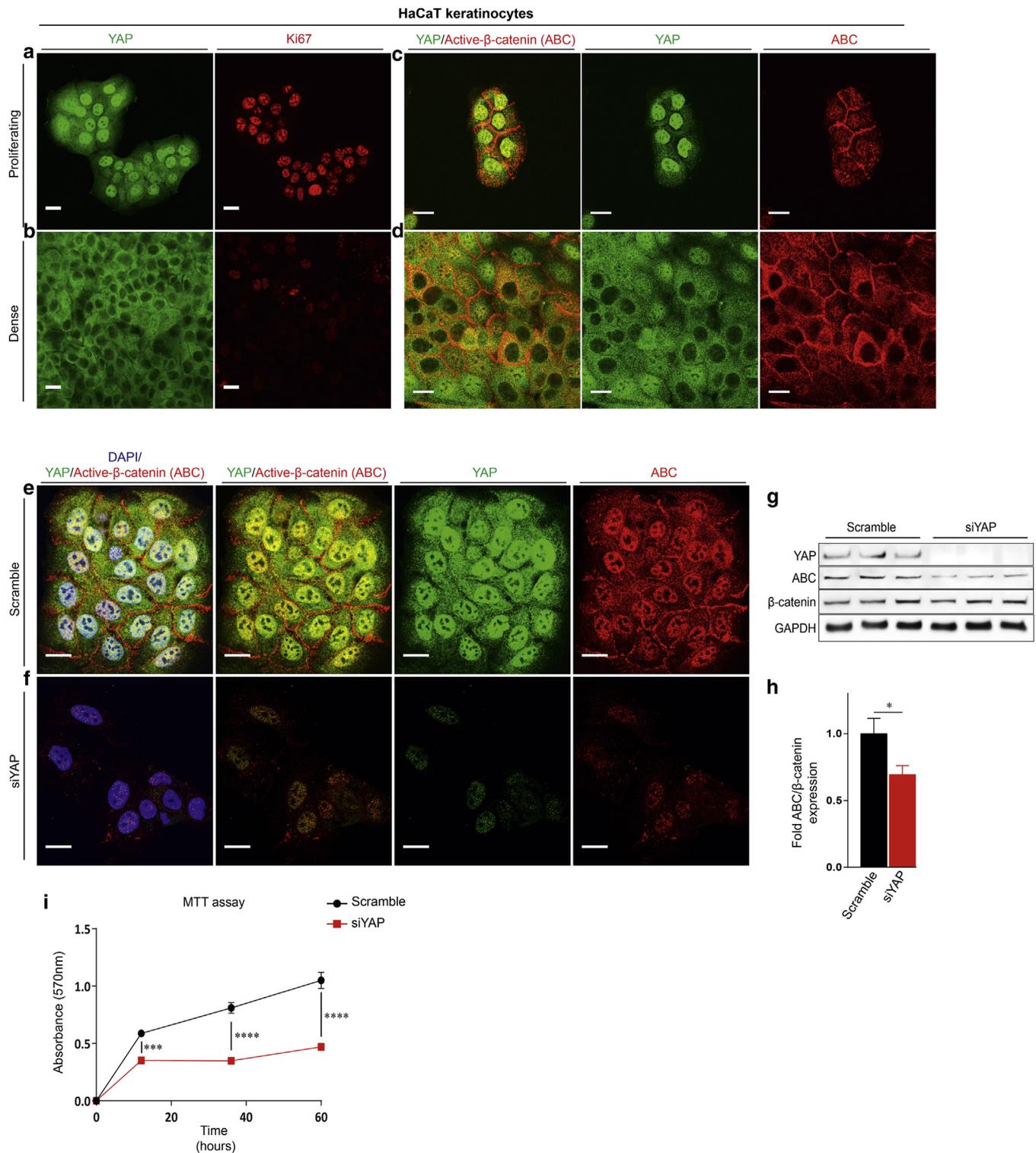


Figure 2. YAP requires β -catenin in controlling proliferation in HaCaT keratinocytes in vitro. Immunofluorescence staining of (a, c) proliferating and (b, d) quiescent HaCaT keratinocytes detecting (a–d) YAP, (a, b) Ki67, and (c, d) active β -catenin. Immunofluorescence staining of (e) scramble RNA and (f) siYAP RNA transfected HaCaT keratinocytes detecting YAP and active β -catenin. (g) Western blots detecting YAP, active β -catenin, total β -catenin, and GAPDH in protein lysates of scramble and siYAP RNA transfected HaCaT keratinocytes. (h) Quantification of active β -catenin protein expression normalized to β -catenin expression in protein lysates of scramble RNA and siRNA RNA-transfected HaCaT keratinocytes. (i) MTT proliferation assay using HaCaT keratinocytes transfected with scramble RNA and YAP siRNA. Scale bars = 20 μ m. * P < 0.05; *** P < 0.001; **** P < 0.0001. ABC, active β -catenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; si, small interfering.

genes *Ccnd1* (Shutman et al., 1999), *Axin2* (Jho et al., 2002), and *c-Myc* (He et al., 1998) mRNAs and of cyclin D1 protein were determined in dorsal skin biopsy samples from

P85 wild-type and YAP2-5SA- Δ C littermates by quantitative qRT-PCR and Western blotting assays (Figure 4a–c). *Ccnd1*, *Axin2*, and *c-Myc* mRNA expression levels were increased

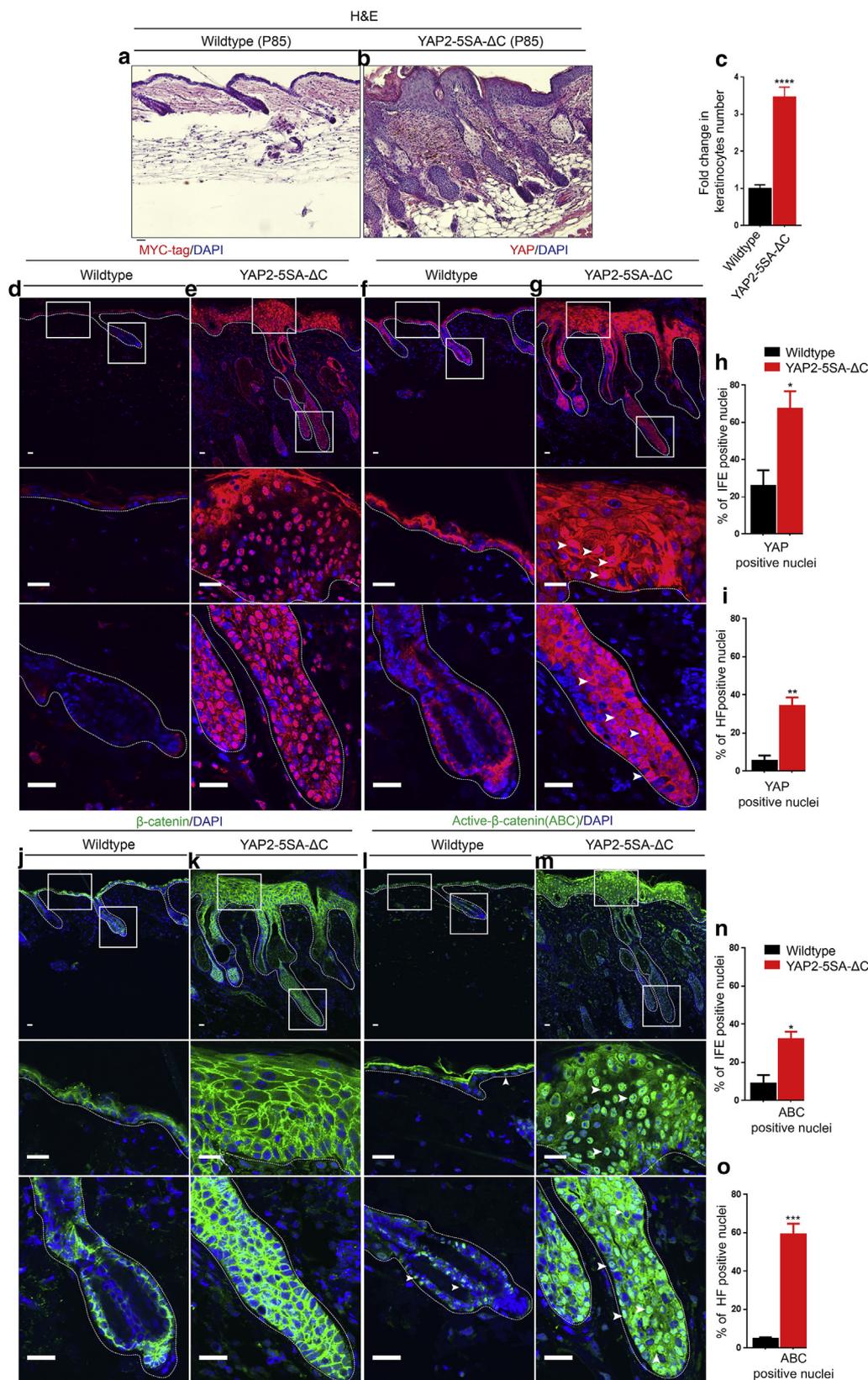


Figure 3. Increased nuclear YAP and β -catenin in keratinocytes of the hyperplastic YAP2-5SA- Δ C epidermis.

(a, b) H&E staining of dorsal skin sections of P85 wild-type and YAP2-5SA- Δ C transgenic littermate mice. (c) Quantification of the fold change in keratinocyte number in the interfollicular epidermis. Immunofluorescence staining of dorsal skin sections of (d, f, j, l) P85 wild-type and (e, g, k, m) YAP2-5SA- Δ C mice detecting (d, e) MYC-YAP2-5SA- Δ C, (f, g) pan-YAP, (j, k) pan- β -catenin, and (l, m) active β -catenin. (h, i) Quantification of percentage pan-YAP and (n, o) percentage active β -catenin-positive nuclei in the interfollicular epidermis and hair follicles of P85 YAP2-5SA- Δ C and wild-type mice. Arrowheads point to (g) pan-YAP and (l, m) active β -catenin-positive nuclei. Scale bars = 20 μ m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. ABC, active β -catenin; H&E, hematoxylin and eosin; HF, hair follicle; IFE, interfollicular epidermis; P, postnatal day.

approximately 1.3- to 2-fold in YAP2-5SA- Δ C relative to wild-type skin ($P < 0.05$, $n = 5$) (Figure 4a), whereas cyclin D1 protein levels were approximately 2-fold higher ($P < 0.01$, $n = 3$) (Figure 4b and c). Immunofluorescence assays showed a

significantly increased percentage of cyclin D1-positive nuclei versus total nuclei in the YAP2-5SA- Δ C versus wild-type epidermis ($P < 0.0001$, $n = 3$) (Figure 4d and e). These data show that YAP2-5SA- Δ C expression in the basal epidermal

cells leads to increased transcription and translation of β -catenin direct target genes in dorsal skin biopsy samples.

To complement these data, β -catenin transcriptional activity in skin of YAP2-5SA- Δ C mice was assessed from postnatal day (P) 50, the onset of visible abnormalities in YAP2-5SA- Δ C mice, until P85, when the hyperplastic abnormalities are very pronounced (Beverdam et al., 2013) using the TOPFLASH mouse line. This is a bioluminescent in vivo reporter system that enables quantitation of β -catenin activity in the skin of live mice at the macroscopic level (Hodgson et al., 2014). YAP2-5SA- Δ C/TOPFLASH double and TOPFLASH single transgenic mice were generated, and bioluminescence levels were acquired from skin of whole mice. Radiance levels varied greatly in the skin of both YAP2-5SA- Δ C/TOPFLASH and TOPFLASH mice, reflecting Wnt signaling activity during hair follicle cycling (Hodgson et al., 2014), but the average radiance was increased by 40% in YAP2-5SA- Δ C/TOPFLASH mice over this time window ($P < 0.01$) (Figure 4g), substantiating our results from dorsal skin biopsy samples (Figures 3l–o and 4a–e).

To better understand the kinetics of β -catenin transcriptional activity in the skin of mice over time, radiance levels were acquired twice weekly from P50 until P85 from four small contiguous skin areas (Figure 4h–m). Levels oscillated in the skin of both YAP2-5SA- Δ C/TOPFLASH and TOPFLASH mice, probably reflecting hair follicle cycling (Hodgson et al., 2014) but reached higher levels in YAP2-5SA- Δ C/TOPFLASH mice (Figure 4h–i) ($P < 0.01$, $n = 4$). In addition, both lowest (Figure 4k) and highest radiance levels (Figure 4m) were significantly higher in the skin of YAP2-5SA- Δ C/TOPFLASH versus TOPFLASH mice ($P < 0.05$, $n = 4$).

The increase in β -catenin transcriptional activity was approximately 16-fold in the skin of YAP2-5SA- Δ C/TOPFLASH versus TOPFLASH mice at baseline β -catenin transcriptional activity levels (Figure 4k). Therefore, this increase in β -catenin transcriptional activity cannot be attributed to the 3- to 4-fold increase in keratinocyte numbers in the YAP2-5SA- Δ C skin at telogen when no active Wnt signaling occurs (Figure 3c), but is caused by epidermal YAP2-5SA- Δ C expression.

Taken together, these data show that β -catenin transcriptional activity is increased in the skin of YAP2-5SA- Δ C mice during the development of the hyperplastic epidermal abnormalities.

Inactivation of β -catenin in basal keratinocytes of YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} mice abolishes the hyperplastic epidermal abnormalities

To assess if β -catenin in the epidermis of YAP2-5SA- Δ C mice was responsible for the increased keratinocyte proliferation, YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{lox/lox} triple mutant mice were generated, and *CtnnB1* was ablated in the basal epidermis by tamoxifen administration at around 3 weeks of age, before onset of the externally evident YAP2-5SA- Δ C phenotype. The epidermal abnormalities were investigated 4–5 weeks later.

PCR genotyping confirmed excision of the floxed regions of both *CtnnB1* alleles (see Supplementary Figure S1 online). Immunofluorescence assays showed that β -catenin expression levels (Figure 5i vs. h) and the number of cyclin

D1-positive nuclei (Figure 5l vs. k and l' and l'') ($P < 0.001$, $n = 3$) were strongly reduced in the YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} versus the YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{lox/lox} epidermis, confirming the successful excision of the conditional *CtnnB1* allele.

Histological analyses showed that the interfollicular epidermis of YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} mice was significantly thinner and the epithelial downgrowths were significantly shorter compared with YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{lox/lox} mice (Figure 5f and f') ($P < 0.0001$, $n = 3$). K14-creERT/*CtnnB1*^{-/-} littermate mice displayed epidermal hyperplasia (see Supplementary Figure S2 online), in line with the initial report by Huelsken et al. (2001), which is thought to occur in response to skin barrier defects (Lim and Nusse, 2013).

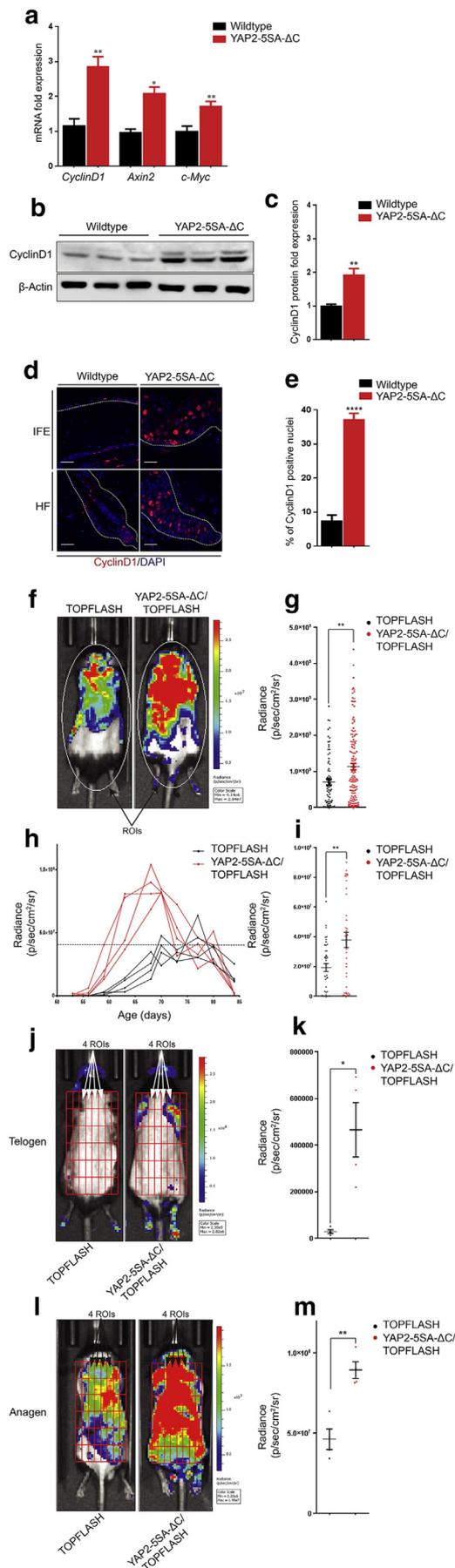
Protein detection analyses showed that the keratin (K) 14-positive basal epidermal layer was thinner in YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} versus YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{lox/lox} skin (Figure 5o vs. n) and displayed reduced K14 protein expression levels (Figure 5p), which were similar to those of phenotypically normal K14-creERT/*CtnnB1*^{lox/lox} skin (Figure 5m and p). Also, the number of P63-positive nuclei in the interfollicular epidermis was significantly reduced ($P < 0.01$, $n = 3$) (Figure 5s vs. r and s'), and the K15 cell population appeared smaller in the YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} hair follicles (Figure 5v vs. u). These data show that the basal epidermal stem/progenitor cell populations were smaller in YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} relative to YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{lox/lox} mice and similar in size to those found in K14-creERT/*CtnnB1*^{lox/lox} control mice (Figure 5q and t). Lastly, a significantly reduced percentage of Ki67-positive nuclei versus total nuclei was detected in the interfollicular epidermis and hair follicles of YAP2-5SA- Δ C/K14-creERT/*CtnnB1*^{-/-} ($P < 0.05$, $n = 3$) (Figure 5y vs. x and y' and y'').

These data show that loss of β -catenin in the basal epidermis of YAP2-5SA- Δ C mice results in reduced keratinocyte cell proliferation and rescue of the hyperplastic abnormalities. These data suggest that YAP positively regulates β -catenin activity to promote basal keratinocyte proliferation during epidermal homeostasis in the mouse skin in vivo.

DISCUSSION

YAP and β -catenin are crucial regulators of stem/progenitor cell proliferation and undergo complex regulatory interactions to coordinate tissue regeneration. Here, we provide evidence that YAP and β -catenin may cooperate in control of HaCaT keratinocyte proliferation in vitro. In strong support of this, we also show that YAP2-5SA- Δ C requires β -catenin transcriptional activity to promote keratinocyte proliferation in the murine epidermis in vivo. Together, our data support the existence of positive regulatory interactions between YAP and β -catenin in the control of epidermal regeneration.

The YAP2-5SA- Δ C protein mutant lacks the YAP C-terminus from Q281, including the C-terminal transactivation and PDZ domains, and it is insensitive to the Hippo pathway-induced cytoplasmic retention in vitro because of mutations in codons encoding its target serine residues (Hoshino et al.,



2006; Zhang et al., 2012; Zhao et al., 2007). Previous reports have shown that it blocks endogenous YAP function in vitro (Hoshino et al., 2006; Zhao et al., 2007). Here, we report predominant nuclear localization of YAP2-5SA- Δ C in the epidermis of YAP2-5SA- Δ C mice (Figure 3e). However, we previously found that YAP2-5SA- Δ C mice display epidermal hyperplasia representing a YAP gain-of-function rather than a YAP loss-of-function phenotype, and we concluded that YAP does not require its C-terminus to drive keratinocyte proliferation in mice in vivo (Beverdam et al., 2013), in line with findings reported in other publications (Dong et al., 2007; Hilman and Gat, 2011; Zhang et al., 2012). In addition, we also found that YAP2-5SA- Δ C mice do not develop skin tumors (data not shown), as opposed to the YAPS127A inducible transgenic mouse model (Schlegelmilch et al., 2011; Zhang et al., 2011). Therefore, the effect of the YAP2-5SA- Δ C mutant on the epidermis in vivo is relatively mild compared to that of the YAPS127A mutant. This is likely explained by the loss of the YAP C-terminal (PDZ) domain, critical for YAP-mediated cell transformation and oncogenesis (Shimomura et al., 2014; Zhang et al., 2012). We still see strong nuclear accumulation of YAP2-5SA- Δ C in the mouse epidermis as opposed to a YAP mutant protein with deleted PDZ binding domain in the mouse liver (Shimomura et al., 2014), suggesting that YAP C-terminal sequences other than the PDZ binding domain and/or tissue context-specific differences contribute to the regulation of YAP nucleocytoplasmic shuttling. Even though the function of the YAP2-5SA- Δ C mouse line nevertheless offers a unique opportunity to gain insights into the regulatory processes downstream of YAP activation in control of keratinocyte proliferation and epidermal homeostasis in vivo.

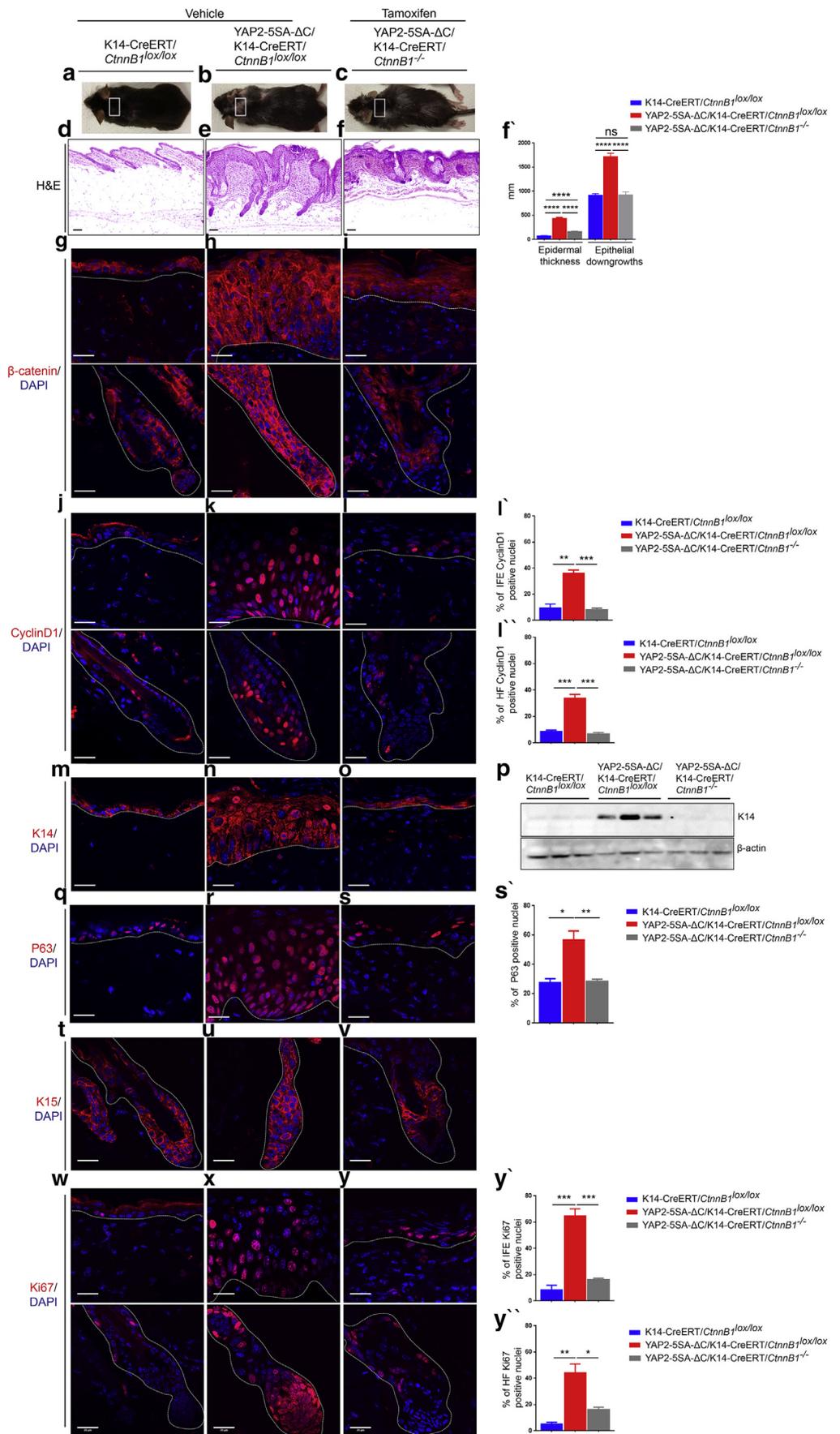
The role of β -catenin in the interfollicular epidermis has long been controversial. Wnt signals have long remained undetectable in the interfollicular epidermis (DasGupta and Fuchs, 1999), and inactivation of β -catenin in K14-positive basal keratinocytes resulted in interfollicular epidermis hyperplasia (Huelsen et al., 2001), which we observed as well in K14-creERT/*CtnnB1*^{-/-} mice (see Supplementary Figure S2), suggesting that β -catenin activation in the interfollicular epidermis may rather repress proliferation. However, this conclusion is controversial, because the abnormal hair follicles in the *CtnnB1* mutant mice may have perturbed

Figure 4. Increased β -catenin transcriptional activity in the skin of YAP2-5SA- Δ C transgenic mice during development of the epidermal hyperplasia.

(a) qRT-PCR quantification of *Ccnd1*, *Axin2*, and *c-Myc* mRNA (n = 5). Cyclin D1 protein in (b, c) protein lysates (n = 3) and (d, e) in sections of dorsal skin of wild-type and YAP2-5SA- Δ C mice. (f–m) Quantification of BLI levels in skin of YAP2-5SA- Δ C/TOPFLASH (n = 17) and TOPFLASH mice (n = 9) during development of the hyperplastic phenotype from P50 until P85. (f, j, l) Representative whole-body BLI emission images. (g) Diagram BLI measurements from P50 until P85 in the skin of YAP2-5SA- Δ C/TOPFLASH and TOPFLASH mice. (h) BLI levels from four contiguous ROIs (arrows in j and l) of a representative YAP2-5SA- Δ C/TOPFLASH and TOPFLASH mouse pair against age. Mean BLI levels at (k) telogen and (m) anagen (n = 4). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. BLI, bioluminescence; HF, hair follicle; IFE, interfollicular epidermis; P, postnatal day; ROI, region of interest. Scale bars = 20 μ m.

Figure 5. Inactivation of β -catenin in basal keratinocytes of YAP2-5SA- Δ C/K14-creERT/*Cttnb1*^{-/-} mice results in alleviation of the hyperplastic epidermal abnormalities.

Phenotypic characterization of (a, d, g, j, m, q, w) P50 K14-creERT/*Cttnb1*^{lox/lox}, (b, e, h, k, n, r, u, x) YAP2-5SA- Δ C/K14-creERT/*Cttnb1*^{lox/lox}, and (c, f, i, l, o, s, v, y) YAP2-5SA- Δ C/K14-creERT/*Cttnb1*^{-/-} mutant mice. (a–c) Representative images of mutant mice with boxed skin area used for analyses. (f) Quantification of epidermal thickness and length of epithelial downgrowths. (d–f) H&E staining. Immunofluorescence analysis detecting (g–i) β -catenin, (j–l) cyclin D1, (m–o) K14, (q–s) p63, (t–v) K15, and (w–y) Ki67. (p) Analysis of K14 expression in skin protein lysates on blots. Quantification of (l', l'') cyclin D1-, (s', s'') P63-, and (y', y'') Ki67-positive nuclei in the interfollicular epidermis and hair follicles. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. H&E, hematoxylin and eosin; HF, hair follicle; IFE, interfollicular epidermis; K, keratin; ns, not significant; Scale bars = 20 μ m.



the skin's barrier function, which generally also leads to epidermal hyperplasia (Lim and Nusse, 2013). Recently, two articles were published that have investigated this discrepancy in detail using highly sensitive in vivo reporter assays, cell-type-specific genetic manipulation and clonal analysis. These researchers showed that Wnt/ β -catenin signaling is active in the interfollicular epidermis and that β -catenin activity is contributing to the proliferation of basal keratinocytes in the interfollicular epidermis (Choi et al., 2013; Lim et al., 2013), which is in line with our own observations (Samuel et al., 2011). Here, we also demonstrate that β -catenin inactivation in basal keratinocytes in the interfollicular epidermis results in decreased proliferation in YAP2-5SA- Δ C/K14-CreER/*Ctnnb1*^{-/-} mice and rescues the epidermal hyperplasia, in strong support of these publications.

It is becoming increasingly clear that the YAP/TAZ/Hippo and β -catenin/Wnt signaling pathways undergo intricate interactions to control stem cell regeneration in a diverse array of tissues and in cancer development (e.g., reviewed in Piccolo et al., 2014; Yu et al., 2015). Recent work has presented evidence for a model in which YAP/TAZ are deeply integrated into the Wnt pathway as components of the β -catenin destruction complex programs dependent on the presence of Wnt signals (Azzolin et al., 2014; Azzolin et al., 2012; Oudhoff et al., 2016). In our studies, expression of the YAP2-5SA- Δ C transgene product is detected mostly in the basal keratinocyte nuclei (Figure 3e), possibly bypassing direct regulatory interactions with β -catenin that may take place in the cytosol. Therefore, even though the exact molecular mechanism of the positive regulatory interactions between YAP and β -catenin that we identified using our YAP2-5SA- Δ C mouse model remains to be established, our data support that other regulatory interactions between these two proteins may take place in addition to those reported in the cytosolic β -catenin destruction complex. Very recent reports do indeed show alternative modes of regulatory interactions between YAP and β -catenin. For instance, Park et al. (2015) recently showed that YAP/TAZ can be downstream effectors of an alternative Wnt signaling pathway via Frizzled, $G\alpha_{12/13}$, Rho GTPases, and LATS1/2 (Park et al., 2015). Also, APC has been shown to act as a scaffolding protein for SAV1 and LATS1, and *Apc* inactivation leads to YAP activation and tumorigenesis (Cai et al., 2015).

What may be the nature of the positive regulatory interactions between activated YAP and β -catenin in control of basal keratinocyte proliferation in the epidermis? It is conceivable that YAP transcriptional activity results in the production of proteins that activate β -catenin cell-autonomously or via a paracrine mechanism, possibly involving Wnt molecules in line with recent findings (Choi et al., 2013; Lim et al., 2013). Conversely, there is evidence that β -catenin and YAP may form a transcriptional complex and share common target genes (Heallen et al., 2011; Rosenbluh et al., 2012; Wang et al., 2014). In addition, a recent report showed that YAP and β -catenin may both act on the cell cycle independently to induce quiescent epithelial cells to proliferate in response to mechanical strain. These authors showed that YAP induced cell cycle re-entry, whereas β -catenin was responsible for cell cycle progression (Benham-Pyle et al., 2015). The true nature of

the molecular interactions between nuclear YAP2-5SA- Δ C and β -catenin in the epidermis remains to be further investigated.

It is unknown if the positive regulatory interaction between nuclear YAP2-5SA- Δ C and β -catenin in stem/progenitor cell proliferation that we describe in the epidermis may be a more generic regulatory mechanism between nuclear YAP and β -catenin that also controls (stem/progenitor) cell proliferation in other tissues. Accumulating evidence shows elevated YAP/TAZ expression and nuclear enrichment in many types of human cancers, including esophageal, liver, breast, lung, colon, ovary, and others, and nuclear localization and high expression of YAP/TAZ target genes are associated with poor outcome (Piccolo et al., 2014; Yu et al., 2015). Conceivably, nuclear YAP drives tumorigenesis through β -catenin nuclear activity in a fashion analogous to the interactions that we describe for YAP2-5SA- Δ C in the epidermis. Our findings may therefore have far-reaching implications for our understanding of the etiology of cancer and other human regenerative disease in tissues displaying increased nuclear YAP and β -catenin activity.

MATERIALS & METHODS

Animals

Animal experimentation was conducted under protocols approved by the UNSW Australia's Animal Care and Ethics Committee Unit and in compliance with the National Health and Medical Research Council's *Australian Code of Practice* (2013). Mouse strains *Ctnnb1*^{lox/lox} (004152) (Baker et al., 2010) and K14-CreERT mice (005107) (Vasioukhin et al., 1999) were obtained from Jackson Laboratories (Bar Harbor, ME). Conditional *Ctnnb1* alleles were excised by daily intraperitoneal injection of 75 mg/kg tamoxifen (Toronto Research Chemicals) for five consecutive days. Genotyping was performed as previously described (Beverdam et al., 2013; Brault et al., 2001; Cattellino et al., 2003; Hodgson et al., 2014; Vasioukhin et al., 1999).

Bioluminescence imaging

Mice were imaged using the IVIS SpectrumCT (Perkin Elmer, Waltham, MA) preclinical in vivo imaging system. Data acquisition and analysis were performed using the Living Image software (Perkin Elmer) as previously described (Hodgson et al., 2014). Data were blindly analyzed by using Student unpaired *t* test with a 2-tailed distribution. *P*-values less than 0.05 were considered statistically significant.

Tissue processing and histological and immunofluorescence staining

Full-thickness skin biopsy samples were processed for paraffin sectioning and histology staining using routine methods. Antigen retrieval was performed using 10 mmol/L sodium citrate buffer (pH 6.0) and a Milestone RHS-1 Microwave (MILESTONE SRL, Italy) at 110 °C for 5 minutes. Sections were immunostained using routine methods, and confocal images were captured using an Olympus FV1200 laser scanning confocal microscope (Olympus, Tokyo, Japan). Immunosignal intensity was quantified in a semiautomated fashion using ImageJ software (National Institutes of Health, Bethesda, MD). Antibody information is available in Table S1 online.

Quantitative RNA and protein expression analysis

Full-thickness skin biopsy samples were homogenized in TRIzol reagent (Life Technologies, Waltham, MA), and RNA and

B Akladios et al.YAP drives β -catenin in Keratinocyte Proliferation

protein were prepared as recommended by the manufacturer (Chomczynski, 1993).

Protein lysates were analyzed on Western blots. Intensity of bands was quantified with ImageJ software and normalized to β -actin. Primary antibodies against the following proteins were used: β -actin (Sigma-Aldrich, St. Louis MO), cyclin D1 (Abcam, Cambridge, UK). Secondary antibodies were donkey anti-rabbit IgG, horseradish peroxidase-conjugated antibody from donkey (Amersham NA934) and mouse IgG, and horseradish peroxidase-conjugated antibody from sheep (Amersham NA931).

qRT-PCR assays were carried out using Fast SYBR Green Master Mix (Life Technologies 4385612) and Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA) and were analyzed by the comparative cycle time method, normalizing to *18S* ribosomal RNA levels. qRT-PCR primers: *Ccnd1*-F: 5'-GAGATTGTGCCATC-CATG C-3', *Ccnd1*-R: 5'-CTCCTCTTCGCACTTCTGCT-3', *Axin2*-F: 5'-GAGAGTGAGCGGCAGAGC-3', *Axin2*-R: 5'-CGGCTGACTCGT TCTCCT-3', *c-Myc*-F: 5'-CCTAGTGCTGCATGAGGAGA-3', *c-Myc*-R: 5'-TCTTCTCATCTTCTTGCTTTC-3', *18S*-F: 5'-GATCCATTG-GAGGGCAAGTCT-3', and *18S*-R: 5'-CCAAGATCCAACACTACGAG CTTTTT-3'.

Cell culture assays

HaCaT immortalized keratinocytes were maintained in DMEM/F-12 (Sigma, D8062), supplemented with 10% FBS (Gibco, 10437-028) and $1 \times$ penicillin-streptomycin (Gibco, Waltham, MA; 15140-122) in a 5% CO₂ incubator at 37 °C. YAP knockdown transient transfections were performed using MISSION Universal and YAP siRNA (Sigma). MTT assays were performed using Thiazolyl Blue Tetrazolium Bromide (Sigma). Immunostaining assays were performed following standard protocols. Antibody information is available in [Supplementary Table S1](#) online. Imaging was performed using an Olympus FluoView FV1200 Confocal Microscope.

Statistical analysis

Statistical significance was determined by Student unpaired *t*-tests. Error bars represent SEM. Asterisks indicate statistical significance, where *P* less than 0.05 was used as the significance cutoff.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.10.029>.

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