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Epidermal YAP2-5SA-ΔC Drives β-Catenin Activation to Promote Keratinocyte Proliferation in the Mouse Skin *in vivo*

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ABSTRACT

The epidermis is a highly regenerative tissue. Yes-associated protein (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-ΔC mice, the mutant YAP2-5SA-ΔC protein was predominantly localised in the keratinocyte nuclei, and caused increased expression of activated, nuclear β-catenin. Accordingly, β-catenin transcriptional activity was elevated in the skin of life YAP2-5SA-ΔC/TOPFLASH mice. Lastly, loss of *β-catenin* in basal keratinocytes of YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{lox/lox}$ mice resulted in reduced proliferation of basal keratinocytes and a striking rescue of the hyperplastic abnormalities.

Taken together, our work shows that YAP-5SA-ΔC requires β-catenin activity to promote basal keratinocyte proliferation in the mouse skin *in vivo*. Our data shines new light on the aetiology of regenerative dermatological disorders, and other human diseases that display increased YAP and β-catenin activity.

INTRODUCTION

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

Yes-associated protein (YAP) and its paralog Transcriptional co-Activator with PDZ-binding motif (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 Transcriptional Enhancer Associate Domain (TEAD) transcription factors to activate target gene transcription and cell proliferation (reviewed in Piccolo et al., 2014, Yu et al., 2015). Recent pioneering work demonstrated 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 demonstrate 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 (Huelsken 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). Interestingly, there is increasing evidence that the Hippo/YAP and Wnt/β-catenin signalling 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, and provide experimental support for the hypothesis that YAP and β-catenin cooperate in the control of HaCaT keratinocyte proliferation *in vitro*. Interestingly, using a viable transgenic mouse line in which the YAP2-5SA-ΔC mutant is predominantly expressed in basal keratinocytes, we found that YAP2-5SA-ΔC requires β-catenin activation to promote basal keratinocyte proliferation in the mouse skin *in vivo*. Together, our findings support 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 wildtype 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 & 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 was 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 previously reported (Zhao et al., 2007), where it co-localized with β-catenin (Figure 2a & c). YAP was predominantly localized in the cytosol of quiescent HaCaT keratinocytes (Figure 2b & d), in line with reported previously (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 siRNA knockdown assays and confirmed a strong reduction of YAP protein expression in YAP siRNA-treated *vs.* scramble RNA-treated HaCaT keratinocytes (Figure 2e, f, and top lane in g). We also observed reduced nuclear activated β-catenin (Figure 2e, f), significantly reduced activated β-catenin *vs*. total β-catenin (Figure 2g & 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 *vs.* 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-ΔC 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-ΔC expression in the basal epidermis on β-catenin was investigated in skin of YAP2-5SA-ΔC transgenic mice (Beverdam et al., 2013). We detected the MYC-tagged YAP2-5SA-ΔC protein predominantly in the nuclei of basal keratinocytes of YAP2-5SA-ΔC skin (Figure 3e). Moreover, we found that YAP2-5SA-ΔC expression in the basal epidermis resulted in increased keratinocyte proliferation, a 3-4-fold increase in keratinocyte numbers (Figure 3c), eventually causing epidermal hyperplasia (Figure 3a & 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-ΔC expression in the basal epidermis of mouse skin *in vivo* promotes keratinocyte proliferation.

Immunofluorescence analysis was performed on dorsal skin of P85 wildtype and YAP2-5SA-ΔC littermates. Relatively high YAP expression levels were detected throughout the hyperplastic epidermis and hair follicles of YAP2-5SA-ΔC littermates, with increased percentage of YAP-positive nuclei *vs.* total nuclei compared to in wildtype skin ($P < 0.05$; *N*=3) (Figure 3h & i). This increased nuclear YAP signal represents YAP2-5SA-ΔC (Figure

3e), further supporting that this mutant YAP protein is mostly localized in keratinocyte nuclei of the YAP2-5SA-ΔC 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 wildtype mice (Figure 3j) and YAP2-5SA-ΔC 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-ΔC (arrowheads in Figure 3m). The percentage of activated β-catenin positive nuclei *vs.* 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-ΔC *vs.* wildtype epidermis (Figure 3n & o). This demonstrates that the increased nuclear accumulation of activated β-catenin cannot be attributed merely to higher keratinocyte numbers in YAP2-5SA-ΔC skin, but occurs due to YAP2-5SA-ΔC presence. These data suggest that YAP2-5SA-ΔC promotes β-catenin activity in the hyperplastic epidermis of YAP2-5SA-ΔC mice *in vivo*.

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

To confirm β-catenin activation in the YAP2-5SA-ΔC epidermis, the expression levels of β-catenin direct target genes *CyclinD1* (Shtutman et al., 1999), *Axin2* (Jho et al., 2002) and *c-Myc* (He et al., 1998) mRNAs and of CyclinD1 protein were determined in dorsal skin biopsies of P85 wildtype and YAP2-5SA-ΔC littermates by quantitative real time reverse transcriptase PCR (qPCR) and Western blotting assays (Figure 4a-c). *CyclinD1*, *Axin2* and *c-Myc* mRNA expression levels were increased approximately 1.3- to 2-fold in YAP2-5SA-ΔC relative to wildtype skin ($P < 0.05$; $N=5$) (Figure 4a), whereas CyclinD1 protein levels were approximately 2-fold higher ($P < 0.01$; $N=3$) (Figure 4b & c). Immunofluorescence

assays showed a significant increased percentage of CyclinD1 positive nuclei *vs*. total nuclei in the YAP2-5SA-ΔC *vs.* wildtype epidermis ($P < 0.0001$; $N=3$) (Figure 4d & e). These data show that YAP2-5SA-ΔC expression in the basal epidermal cells leads to increased transcription and translation of β-catenin direct target genes in dorsal skin biopsies.

To complement this data, β-catenin transcriptional activity in skin of YAP2-5SA-ΔC mice was assessed from P50, the onset of visible abnormalities in YAP2-5SA-ΔC 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-ΔC/TOPFLASH double and TOPFLASH single transgenic mice were generated, and bioluminescence levels were acquired from skin of whole mice. Radiance levels varied greatly in skin of both YAP2-5SA-ΔC/TOPFLASH and TOPFLASH mice, reflecting Wnt signalling activity during hair follicle cycling (Hodgson et al., 2014), but the average radiance was increased by 40% in YAP2-5SA-ΔC/TOPFLASH mice over this time window ($P < 0.01$) (Figure 4g), substantiating our results from dorsal skin biopsies (Figure 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 4 small contiguous skin areas (Figure 4h-m). Levels oscillated both in skin of YAP2-5SA-ΔC/TOPFLASH and TOPFLASH mice, probably reflecting hair follicle cycling (Hodgson et al., 2014), but reached higher levels in YAP2-5SA-ΔC/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 skin of YAP2-5SA-ΔC/TOPFLASH *vs.* TOPFLASH mice ($P < 0.05$, $N=4$).

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

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

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

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

PCR-genotyping confirmed excision of the floxed regions of both *β-catenin* alleles (Figure S1). Immunofluorescence assays revealed that β-catenin expression levels (Figure 5i *vs.* h) and the number of CyclinD1 positive nuclei (Figure 5l *vs.* k & l`& l``, $P < 0.001$, $N = 3$) were strongly reduced in the YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ *vs.* the YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{lox/lox}$ epidermis, confirming the successful excision of the conditional *β-catenin* allele.

Histological analyses showed that the interfollicular epidermis of YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ mice was significantly thinner, and the epithelial down growths were significantly shorter compared to in YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{lox/lox}$ mice (Figure

5f, f'; $P < 0.0001$, $N=3$). K14-creERT/*CtnnB1*$^{-/-}$ littermate mice displayed epidermal hyperplasia (Figure S2) 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 revealed that the K14-positive basal epidermal layer was thinner in YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ *vs.* YAP2-5SA-ΔC/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 & p). Also the number of P63-positive nuclei in the interfollicular epidermis was significantly reduced ($P < 0.01$, $N = 3$) (Figure 5s *vs.* r & s`), and the K15 cell population appeared smaller in the YAP2-5SA-ΔC/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-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ relative to YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{lox/lox}$ mice, and similar in size to those found in K14-creERT/*CtnnB1*$^{lox/lox}$ control mice (Figure 5q & t). Lastly, a significantly reduced percentage of Ki67-positive nuclei *vs*. total nuclei was detected in the interfollicular epidermis and hair follicles of YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ ($P < 0.05$, $N = 3$) (Figure 5y *vs.* x & y` & y``).

These data demonstrate that loss of β-catenin in the basal epidermis of YAP2-5SA-ΔC 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 demonstrate that YAP2-5SA-ΔC requires β-catenin transcriptional activity to promote keratinocyte proliferation in the murine epidermis *in vivo*. Together, our data are in support of the existence of positive regulatory interactions between YAP and β-catenin in the control of epidermal regeneration.

The YAP2-5SA-ΔC 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-ΔC in the epidermis of YAP2-5SA-ΔC mice (Figure 3e). However, we previously found that YAP2-5SA-ΔC mice display epidermal hyperplasia rather representing a YAP 'gain-of-function' 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-ΔC mice do not develop skin tumors (not shown) as opposed to the YAPS127A inducibile transgenic mouse model (Schlegelmilch et al., 2011, Zhang et al., 2011). Therefore, the effect of the YAP2-5SA-ΔC 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). Interestingly, we still see

strong nuclear accumulation of YAP2-5SA-ΔC 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-ΔC protein mutant is not fully understood, the YAP2-5SA-ΔC 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 (Huelsken et al., 2001), which we observed as well in K14-creERT/*CtnnB1*$^{-/-}$ mice (Figure S2), suggesting that β-catenin activation in the interfollicular epidermis may rather repress proliferation. However, this conclusion is controversial, as the abnormal hair follicles in the *β-catenin* mutant mice may have perturbed the skin's barrier function, which generally also leads to epidermal hyperplasia (Lim and Nusse, 2013). Recently, two papers 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 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-ΔC/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 signalling 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 where 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-ΔC transgene product is detected mostly in the basal keratinocyte nuclei (Figure 3e), possibly by-passing direct regulatory interactions with β-catenin that may take place in the cytosol. So even though the exact molecular mechanism of the positive regulatory interactions between YAP and β-catenin that we identified using our YAP2-5SA-ΔC 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 yet 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 signalling 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).

So 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-ΔC and β-catenin in the epidermis remains to be further investigated.

It is unknown if the positive regulatory interaction between nuclear YAP2-5SA-ΔC 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-ΔC 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.

MATERIAL & 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 'Australian code of practice (8th edition, 2013). Mouse strains *CtnnB1*$^{lox/lox}$ (004152) (Baker et al., 2010) and K14-CreERT mice (005107) (Vasioukhin et al., 1999) were obtained from the Jackson Laboratories. Conditional *β-catenin* alleles were excised by daily intraperitoneal injection of 75 mg/kg Tamoxifen (TRC) for five consecutive days. Genotyping was performed as previously described (Beverdam et al., 2013, Brault et al., 2001, Cattelino et al., 2003, Hodgson et al., 2014, Vasioukhin et al., 1999).

Bioluminescence imaging

Mice were imaged using IVIS® SpectrumCT (Perkin Elmer) pre-clinical *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 analysed by using Student's unpaired *t*-test with a 2-tailed distribution. *P*-values < 0.05 were considered statistically significant.

Tissue processing and Histological and Immunofluorescence staining

Full thickness skin biopsies were processed for paraffin sectioning and histology staining using routine methods. Antigen retrieval was performed using 10mM sodium citrate buffer (pH 6.0) and a Milestone RHS-1 Microwave 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. Immuno-signal intensity was quantified in a semi-automated fashion using ImageJ software. Antibody information is available in Table S1.

Quantitative RNA and Protein expression analysis

Full thickness skin biopsies were homogenized in TRIzol® reagent (Life Technologies), and RNA and protein were prepared as recommended by the manufacturer (Chomczynski, 1993).

Protein lysates were analysed 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), CyclinD1 (Abcam). Secondary antibodies: donkey anti-rabbit IgG, HRP-conjugated antibody, from donkey (Amersham NA934) and mouse IgG, HRP-conjugated antibody, from sheep (Amersham NA931).

Quantitative real-time reverse transcriptase–PCR assays were carried out using Fast SYBR® Green Master Mix (Life Technologies 4385612) and Mx3000P qPCR System (Agilent Technologies), and were analysed by the comparative cycle time method, normalizing to *18S* ribosomal RNA levels. Quantitative real-time reverse transcriptase–PCR primers: *CyclinD1*-F: 5'-GAGATTGTGCCATCCATG C-3', *CyclinD1*-R: 5'-CTCCTCTTCGCACTTCTGCT-3', *Axin2*-F: 5'-GAGAGTGAGCGGCAGAGC-3', *Axin2*-R: 5'-CGGCTGACTCGTTCTCCT-3', *c-Myc*-F: 5'-CCTAGTGCTGCATGAGGAGA-3', *c-Myc*-R: 5'-TCTTCCTCATCTTCTTGCTCTTC-3', *18S*-F: 5'-GATCCATTGGAGGGCAAGTCT-3' and *18S-R*: 5'-CCAAGATCCAACTACGAGCTTTTT-3'.

Cell culture assays

HaCaT immortalized keratinocytes were maintained in DMEM/F-12 (Sigma, D8062), supplemented with 10% FBS (Gibco, 10437-028) and 1X Penicillin-Streptomycin (Gibco, 15140-122) in a 5% CO_2 incubator at 37ºC. 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 Table S1. Imaging was performed using an Olympus FluoView™ FV1200 Confocal Microscope.

Statistical analysis

Statistical significance was determined by Student's unpaired *t*-tests. Error bars represent mean ± SEM. Asterisks indicate statistical significance, where $P < 0.05$ was used as significance cut-off.

CONFLICT OF INTEREST

The authors have no conflicts to declare.

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FIGURE LEGENDS

Figure 1. YAP and β-catenin co-localize in the regenerating epidermis *in vivo*.

Immunofluorescence staining of dorsal skin sections at telogen (a-e) and anagen (f-j) of adult wildtype mice detecting YAP (a, b, f & g), pan β-catenin (a, c, f & h), Keratin 14 (d & i) and Ki67 (e & j). Arrowheads point to YAP (a, b, f & g) and β-catenin positive nuclei (a, c, f & h). Basement membranes and hair follicles are demarcated with dashed lines. P, postnatal day; DAPI, 4, 6-diamidino-2-phenylindole. Scale bars = 20 μm.

Figure 2. YAP requires β-catenin in controlling proliferation in HaCaT keratinocytes *in vitro*.

Immunofluorescence staining of proliferating (a & c) and quiescent (b & d) HaCaT keratinocytes detecting YAP (a-d), Ki67 (a & b), active β-catenin (c & d). Immunofluorescence staining of scramble RNA (e) and siYAP RNA (f) 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. Quantification of active-β-catenin protein expression normalized to β-catenin expression in protein lysates of scramble RNA and siRNA RNA transfected HaCaT keratinocytes (h). MTT proliferation assay using HaCaT keratinocytes transfected with scramble RNA and YAP siRNA (i). DAPI, 4, 6-diamidino-2-phenylindole. Scale bars = 20 μm.

Figure 3. Increased nuclear YAP and β-Catenin in keratinocytes of the hyperplastic YAP2-5SA-ΔC epidermis.

(a & b) H&E staining of dorsal skin sections of P85 wildtype and YAP2-5SA-ΔC transgenic littermate mice. (c) Quantification of the fold change in keratinocyte number in the interfollicular epidermis. Immunofluorescence staining of dorsal skin sections of P85

wildtype (d, f, j & l) and YAP2-5SA-ΔC (e, g, k & m) mice detecting MYC-YAP2-5S-ΔC (d & e), pan-YAP (f & g), pan-β-catenin (j & k) and active-β-catenin (l & m). Quantification of % pan-YAP (h & i) and % active-β-catenin (n & o) positive nuclei in the interfollicular epidermis and hair follicles of P85 YAP2-5S-ΔC and wildtype mice. Arrowheads point to pan-YAP (g) and active-β-catenin positive nuclei (l & m). P, postnatal day; ABC, active β-catenin; DAPI, 4, 6-diamidino-2-phenylindole. Scale bars = 20 µm.

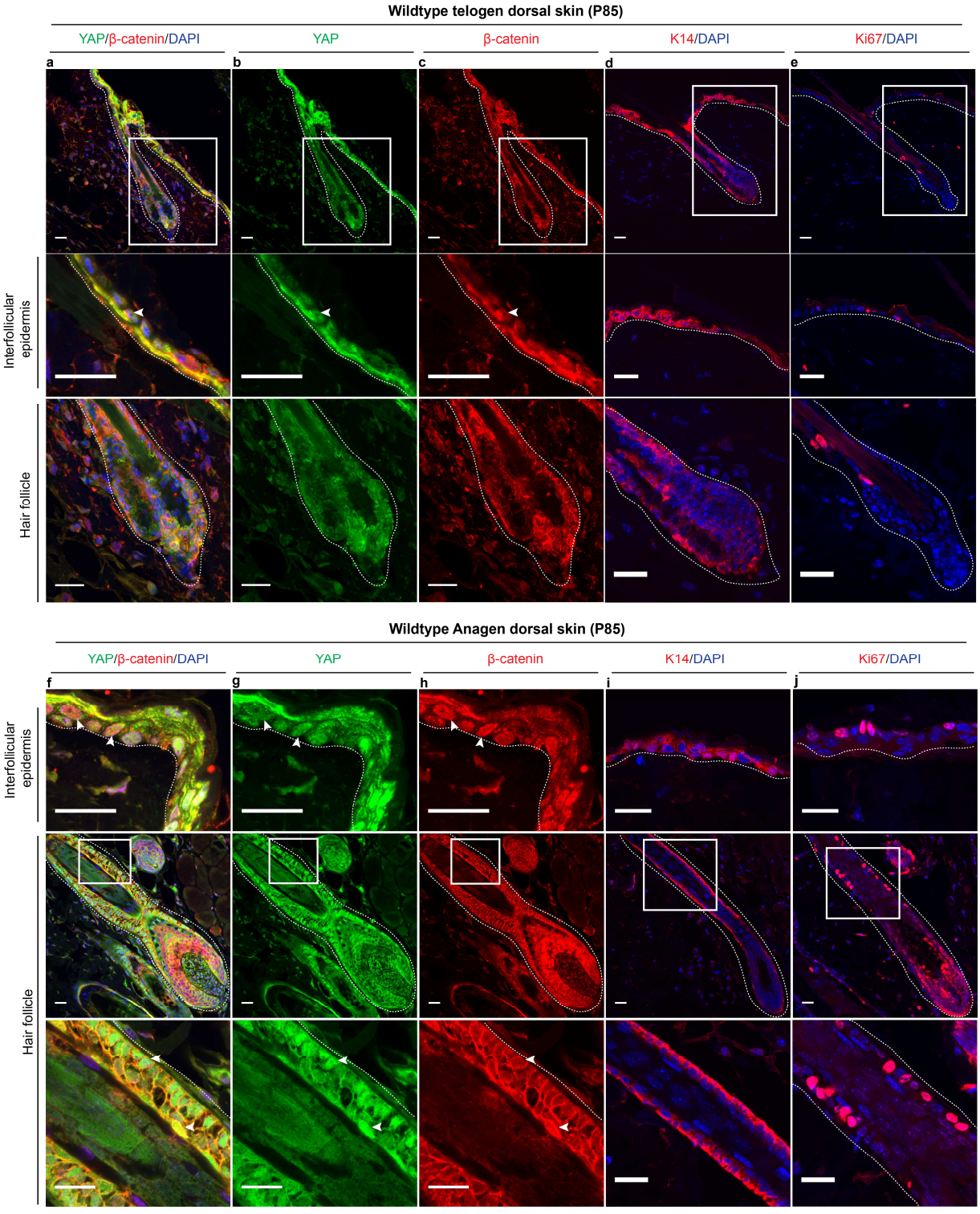
Figure 4. Increased β-catenin transcriptional activity in the skin of YAP2-5SA-ΔC transgenic mice during development of the epidermal hyperplasia.

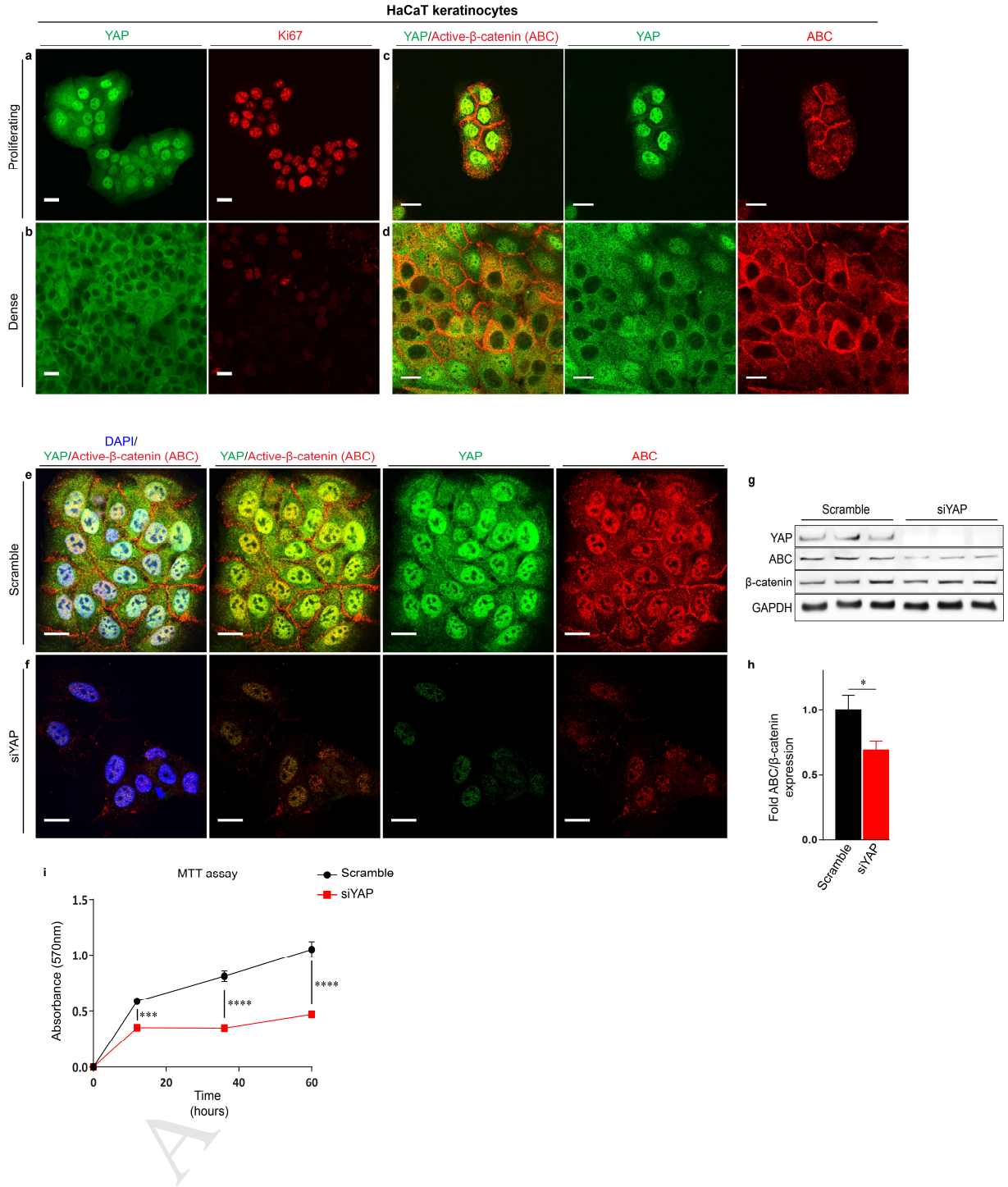
qPCR quantification of *CyclinD1*, *Axin2* and *c-Myc* mRNA (*N*=5; a), CyclinD1 protein in protein lysates (*N*=3; b-c) and in sections (d-e) of dorsal skin of wildtype and YAP2-5SA-ΔC mice. Quantification of BLI levels in skin of YAP2-5SA-ΔC/TOPFLASH (*N*=17) and TOPFLASH mice (*N*=9) during development of the hyperplastic phenotype from P50 till P85 (f-m). (f, j & l) Representative whole-body BLI emission images. (g) Diagram BLI measurements from P50 till P85 in skin of YAP2-5SA-ΔC/TOPFLASH and TOPFLASH mice. (h) BLI levels from 4 contiguous ROIs (arrows in j, l) of a representative YAP2-5SA-ΔC/TOPFLASH and TOPFLASH mouse pair against age. Mean BLI levels at telogen (k) and anagen (m) (*N*=4). mRNA, messenger RNA; BLI, bioluminescence; ROI, region of interest; DAPI, 40,6-diamidino-2-phenylindole. Scale bars = 20 µm.

Figure 5. Inactivation of *β-catenin* in basal keratinocytes of YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ mice results in alleviation of the hyperplastic epidermal abnormalities

Phenotypic characterization of P50 K14-creERT/*CtnnB1*$^{lox/lox}$ (a, d, g, j, m, q, & w), YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{lox/lox}$ (b, e, h, k, n, r, u & x), and YAP2-5SA-ΔC/K14-creERT/*CtnnB1*$^{-/-}$ (c, f, i, l, o, s, v & y) mutant mice. Representative images of mutant mice with boxed skin area used for analyses (a-c). (f`) Quantification of epidermal thickness and length of epithelial downgrowths. H&E staining (d-f). Immunofluorescence analysis

detecting β-catenin (g-i), Cyclin D1 (j-l), K14 (m-o), p63 (q-s), K15 (t-v) and Ki67 (w-y). Analysis of K14 expression in skin protein lysates on blots (p). Quantification of CyclinD1 (l` & l``) and P63 (s`) and Ki67 (y` & y``) positive nuclei in the interfollicular epidermis and hair follicles. H&E, Hematoxylin and eosin; DAPI, 4, 6-diamidino-2-phenylindole. Scale bars = 20 µm.




HaCaT keratinocytes
YAP
Ki67
YAP/Active-β-catenin (ABC)
YAP
ABC
a
b
c
d
Proliferating
Dense
DAPI/
YAP/Active-β-catenin (ABC)
YAP/Active-β-catenin (ABC)
YAP
ABC
e
f
Scramble
siYAP
g
Scramble
siYAP
YAP
ABC
β-catenin
GAPDH
h
Fold ABC/β-catenin expression
1.0
0.5
0.0
*
Scramble
siYAP
i
MTT assay
Scramble
siYAP
Absorbance (570nm)
1.5
1.0
0.5
0.0
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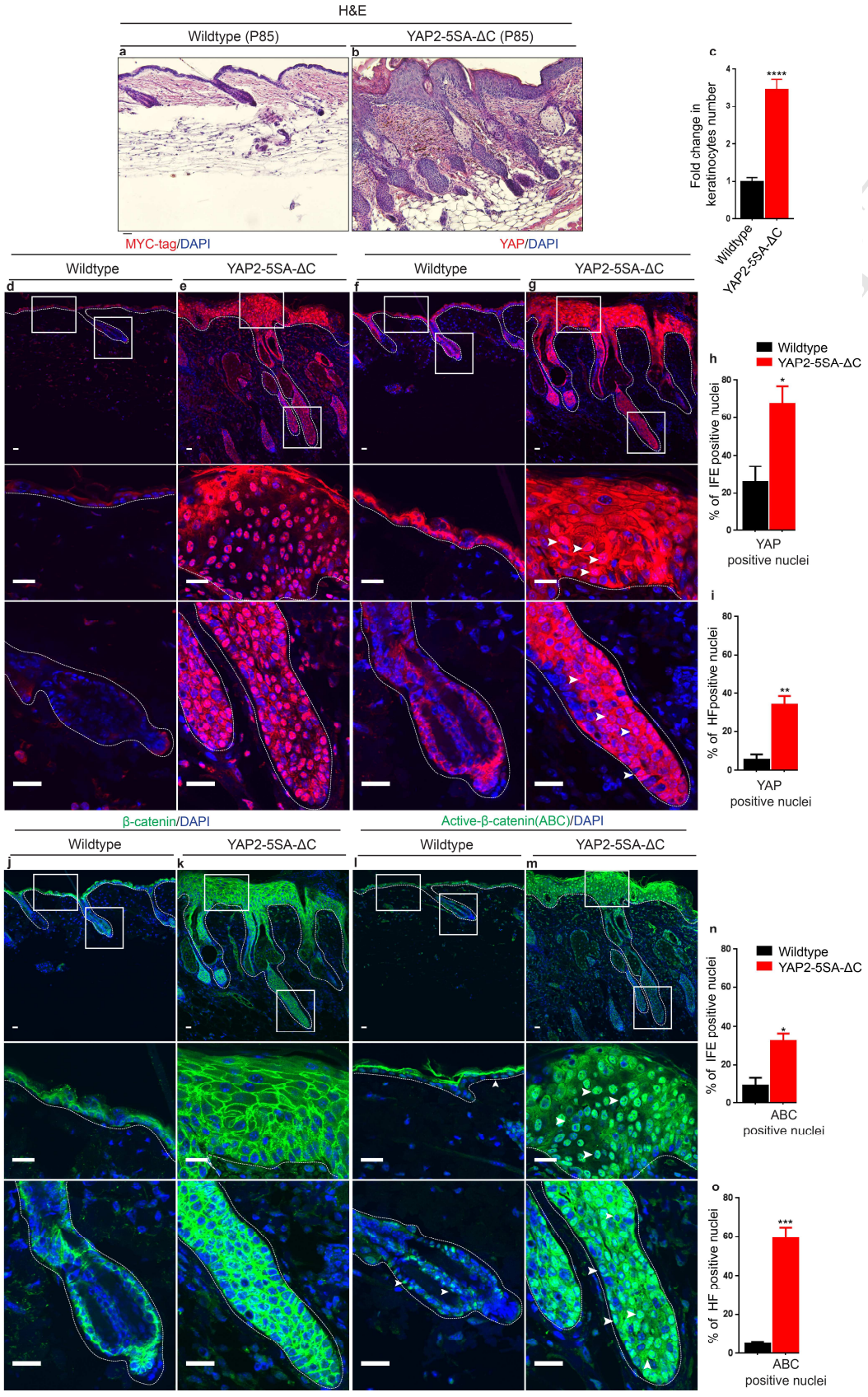
0
20
40
60
Time
(hours)


H&E
Wildtype (P85)
YAP2-5SA-ΔC (P85)
a
b
c
Fold change in keratinocytes number
****
Wildtype
YAP2-5SA-ΔC
MYC-tag/DAPI
YAP/DAPI
Wildtype
YAP2-5SA-ΔC
Wildtype
YAP2-5SA-ΔC
d
e
f
g
h
Wildtype
YAP2-5SA-ΔC
% of IFE positive nuclei
*
YAP
positive nuclei
i
% of HFpositive nuclei
**
YAP
positive nuclei
β-catenin/DAPI
Active-β-catenin(ABC)/DAPI
Wildtype
YAP2-5SA-ΔC
Wildtype
YAP2-5SA-ΔC
j
k
l
m
n
Wildtype
YAP2-5SA-ΔC
% of IFE positive nuclei
*
ABC
positive nuclei
o
% of HF positive nuclei
***
ABC
positive nuclei