YAP1 and the Hippo Signaling Pathway Regulate Progenitor Proliferation

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1. Introduction

1.1 The Hippo Signaling Pathway

Looking at the different species of the animal kingdom, it is striking to see such an immense variation in organism size. An elephant, for example, grows to be a million times larger than a mouse. Additionally, the internal organs of an organism receive information telling them to grow proportionally to the overall body size. The mechanisms directing these growth regulatory processes of organisms and organs fascinate biologists all over the globe, however, they are still poorly understood. A better understanding of growth regulatory mechanisms might ultimately lead to the development of better medical treatments for human diseases associated with abnormal proliferation, such as cancer. An interesting organ in terms of growth potential is the liver, which is able to regenerate its original mass, even when two-thirds of it had been removed by hepatectomy. It is unclear, however, how it senses when to stop growing (Michalopoulos and DeFrances, 1997).

Several studies were conducted to obtain more information about these signals and the results suggest that the information regulating organ growth can be intrinsic to the organ as well as extrinsic. When juvenile organs such as kidney, thymus, or the growth plate were transplanted into adult recipients, these organs retained their growth potential until they had reached their adult sizes, arguing for a growth-sensor intrinsic to the organ (Metcalf, 1963; Stevens et al., 1999; Pape et al., 2006). In contrast, a long-standing theory suggests, that soluble factors secreted by cells, so-called chalones, are responsible for organ growth (Bullough and Laurence, 1964). Recent transplantation studies support the theory that information regulating the growth process is obtained extrinsically through circulating factors. In an experiment where livers were transplanted from small dogs to large dogs, the transplanted livers grew until they had reached the size expected for the recipient (Kam et al., 1987). It had been previously demonstrated that a hepatectomy performed in one rat joined to another rat by parabiosis led to stimulation of hepatocyte proliferation in both animals (Moolten and Bucher, 1967). More recent parabiosis experiments connecting the vascular system of young and old mice showed that factors of the young organism were able to increase the proliferation capacity of muscle satellite cells and hepatocytes in the older mice (Conboy et al., 2005).

Stem cells are a prerequisite for organ growth and have been shown to affect the final size of certain organs when their amount or activity is manipulated (Depaepe et al., 2005; Stanger et al., 2007). Though numerous signaling molecules are known to regulate cell proliferation, it is unclear how the information about organ size is integrated and translated to result in stem cell proliferation and apoptosis. Genetic screens in *Drosophila melanogaster* have led to the discovery of a novel signaling network, the Hippo signaling pathway, that is able to restrict tissue growth in the fly by limiting cell proliferation and promoting apoptosis, thereby determining organ size. Interestingly, it was demonstrated later that this pathway is highly conserved in mammals, which led to novel discoveries regarding organ growth regulation in mammals.

1.1.1 The Hippo Signaling Pathway Core Kinase Cascade

In genetic screens aimed at identifying tumor suppressor genes in *Drosophila melanogaster*, the kinase Warts (wts) was identified in 1995 as the first component of the Hippo signaling pathway (Justice et al., 1995; Xu et al., 1995). In subsequent studies three additional tumor suppressor genes were identified: the kinase Hippo (Hpo), which gave the pathway its name, and the scaffolding proteins Salvador (Sav) and Mob as tumor suppressor (Mats) (Kango-Singh, 2002; Tapon et al., 2002; Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003; Lai et al., 2005). These four components form the core protein cascade of the Hippo signaling pathway with the Hpo-Sav kinase complex phosphorylating and thus activating the Wts-Mats kinase complex (Figure 1; Wu et al., 2003; Huang et al., 2005; Wei et al., 2007).

The downstream effector of this pathway, the transcriptional coactivator Yorkie (Yki) was discovered in a yeast two-hybrid screen for Wts-interacting proteins (Huang et al., 2005). It was demonstrated that Wts directly phosphorylates Yki on serine residue S168 leading to 14-3-3 protein binding, which renders Yki inactive in the cytoplasm (Dong et al., 2007; Zhao et al., 2007; Oh and Irvine, 2008). In its unphosphorylated state, the transcriptional coregulator Yki translocates to the nucleus, where it interacts with the transcription factor Scalloped (Sd) (Wu et al., 2008; Zhang et al., 2008b). Loss-of-function mutations for Hpo, Sav, Wts and Mats cause strong tissue overgrowth phenotypes in the fly, that are characterized by enhanced proliferation and reduced apoptosis (Kango-Singh, 2002; Tapon et al., 2002; Harvey et al.,

2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Lai et al., 2005). Similar phenotypes are observed in gain-of-function mutations for Yki, in line with Yki being the downstream target of the Hippo cascade (Huang et al., 2005).

In mammals the core set of Hippo signaling components is highly conserved. The mammalian orthologs MST1/2 (Hpo), SAV1 (Sav), LATS1/2 (Wts), MOB1 (Mats) and YAP/TAZ (Yki) form a kinase cascade analogous to their fly counterparts (Figure 1; Chan et al., 2005; Zhao et al., 2007). In mammalian cells nuclear YAP induces growth, as observed for Yki in the fly (Zhao et al., 2007). The evolutionary conservation is further underscored by the finding that human MST2, MOB, LATS1 and YAP are able to rescue the loss-of-functions mutant phenotypes of their corresponding Drosophila counterparts *in vivo* (Tao et al., 1999; Wu et al., 2003; Huang et al., 2005; Lai et al., 2005).

Figure 1 presents an overview of the Hippo signaling pathway in *Drosophila melanogaster* and mammals. The Sterile 20-like kinases MST1 and MST2 and their regulatory protein SAV1 (also known as WW45) interact to form an activated complex. In mammalian cells the Ras association domain family (RASSF) proteins can activate MST1/2 (Khokhlatchev et al., 2002; Oh et al., 2006; Guo et al., 2007; 2011). Interestingly, in *Drosophila*, dRASSF instead inactivates Hpo (Polesello et al., 2006). Activated MST1/2 can directly phosphorylate and thus activate the large tumor suppressor homolog kinases LATS1 and LATS2 and their regulatory proteins MOB (MOBKL1A/B) (Chan et al., 2005; Dong et al., 2007; Hirabayashi et al., 2008; Chow et al., 2010). Activated LATS1/2 phosphorylate the transcriptional coactivators YAP at S127 and TAZ at S89 in humans, or S112 and S87 in mouse YAP and TAZ respectively. Phosphorylation at these residues leads to the cytoplasmic retention and thus inactivation of YAP and TAZ through 14-3-3 binding (Dong et al., 2007; Zhao et al., 2007; Hao et al., 2008; Lei et al., 2008; Oh and Irvine, 2008; Oka et al., 2008; Zhang et al., 2008a). When unphosphorylated, YAP and TAZ translocate to the nucleus where they interact with different transcription factors, many of which have been implicated in stem cell biology (Mauviel et al., 2011). However, the group of transcription factors that has been described best to mediate YAP and TAZ activities belongs to the TEA-domain family (TEAD, also known as transcription enhancer factors (TEFs), which consists of four family members TEAD1-4 (Zhao et al., 2008; Zhang et al., 2009a).

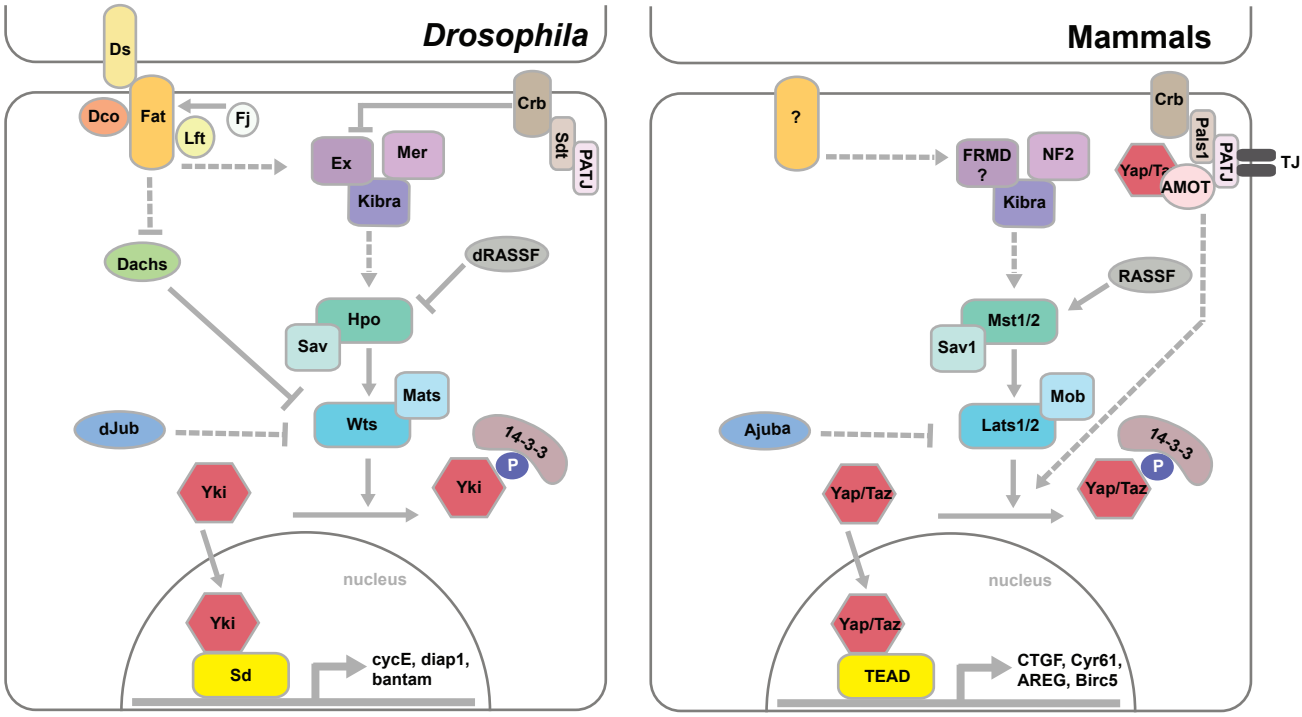

Figure 1: Hippo Signaling Pathway in *Drosophila* and Mammals.

Corresponding components in *Drosophila* and mammals are shown in the same color. ***Drosophila***: In a protein kinase cascade, the Hippo (Hpo) - Salvador (Sav) complex phosphorylates and activates the Warts (Wts) - Mob1 as tumor suppressor (Mats) complex, which in turn phosphorylates the downstream effector, transcriptional coactivator Yorkie (Yki), and thus creates a 14-3-3 binding site that leads to the cytoplasmic localization of Yki. **Mammals**: In a protein kinase cascade, the Mst1/2-Sav1 complex phosphorylates and activates Large tumor suppressor 1/2 (Lats1/2) – Mob complex, which then phosphorylates transcriptional coactivator Yes-associated protein (Yap) and Taz, and thus creates a 14-3-3 binding site that leads to retention of Yap and Taz in the cytoplasma. The genes induced by Yki and Yap are not identical. In mammals the best-studied target genes are the connective tissue growth factor (CTGF) and Cystein-rich protein (Cyr61).

Abbreviations for other components involved: Crumbs (Crb), Dachsous (Ds), Discs overgrown (Dco), Expanded (Ex), Four-jointed (Fj), FERM-containing protein (FRMD), Low-fat (Lft), Merlin (Mer), Neurofibromatosis type 2 gene (NF2), Pals1-associated TJ protein (PATJ), tight junction (TJ) and Stardust (Sdt). Dashed arrows indicate unknown biochemical mechanisms and question marks present unknown components. Pointed and blunt arrowheads indicate activating and inhibitory interactions, respectively.

1.1.2 YAP

The Yes-associated protein (YAP) was originally cloned as an interaction partner of the non-receptor tyrosine kinase c-Yes, which YAP binds at the SH3 domain. Today, YAP is primarily recognized as the major downstream target of the Hippo signaling pathway (Sudol, 1994; Sudol et al., 1995). The gene for *Yap1* is located on the human chromosome 11q22 locus. The *Yap1* gene generates two major protein isoforms, YAP1 (454aa) and the longer isoform YAP2 (488aa), which differ only in the number of WW domains. YAP1 contains one WW domain whereas YAP2 contains two. In this work YAP will be used to describe YAP1/YAP2, and the specific YAP isoforms will be indicated if applicable. Figure 2 shows the human YAP2 protein and its structural domains. The WW domains are important for the interaction between YAP and LATS1/2. The serine residue S127 gets phosphorylated by LATS1/2, and serves as a binding site for 14-3-3 proteins (Basu et al., 2003; Zhao et al., 2007). LATS1/2 also phosphorylate the serine residues S61, S109, S164, and S381 of YAP2 (Zhao et al., 2010).

Of these serine residues, two are known to be important for YAP inactivation. One is S127, whose phosphorylation results in the cytoplasmic localization of YAP, rendering it inactive through spatial regulation. The other is S381 (YAP2), where LATS1/2 phosphorylation causes casein kinase 1 (CK1δ/ε) to phosphorylate two additional serine residues (S384 and S387) located within a phosphodegron sequence. Activation of the phosphodegron results in the ubiquitination of YAP by the $SCF^{\beta\text{-}TRCP}$ E3 ubiquitin ligase, similar to that observed for β-catenin. Ubiquitination targets YAP for degradation, thus providing a means for temporal regulation (Liu et al., 2002; Fuchs et al., 2004; Zhao et al., 2010). It is not only serine residues that play a role in the regulation of YAP. One study showed that the phosphorylation of YAP on Tyrosine Y357 (YAP1) or Y391 (YAP2) by c-Abl in response to DNA damage leads to YAP stabilization and promotes p73-mediated apoptosis. This indicates a possible mechanism limiting the oncogenic potential of YAP under certain circumstances (Levy et al., 2008).

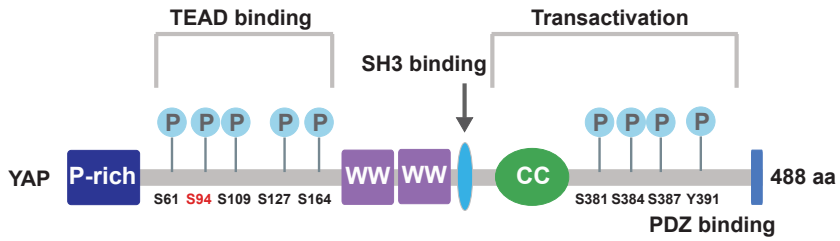

Figure 2: Domain Structure of Human YAP2. YAP2 contains a proline-rich region (PR), two tandem WW domains, a coiled-coil domain (CC) and a PDZ binding motif. Serine residues phosphorylated by LATS1/2 are S61, S109, S127, S164 and S381. Y391 is phosphorylated by c-Abl. CK1δ/ε phosphorylates S384 and S387, which targets YAP2 for degradation. S94 (in red) is crucial for the interaction with the TEAD family transcription factors.

Another very important serine residue in human YAP is S94 (S79 in mouse). It has been demonstrated that S94 is crucial for the interaction of YAP with its main transcription factors of the TEAD family. When S94 is mutated, YAP can no longer bind to the TEADs, greatly diminishing the pro-proliferative activities of YAP (Zhao et al., 2008).

1.1.3 Downstream Regulators

YAP and TAZ are co-transcriptional activators and cannot directly bind to DNA. Therefore, in order to stimulate gene expression, they must bind to DNA-binding transcription factors. YAP has been shown to interact with multiple transcription factors, such as p73, Runx2, Smad7 and the TEAD family transcription factors (Yagi et al., 1999; Strano et al., 2001; Vassilev et al., 2001; Ferrigno et al., 2002; Zaidi et al., 2004). Additional transcription factors, such as PAX3 and SMAD2/3/4, interact with YAP's ortholog TAZ (Murakami et al., 2006; Varelas et al., 2008). However, YAP and TAZ primarily relay the information of the Hippo signaling pathway through interaction with the TEADs (Ota and Sasaki, 2008; Zhao et al., 2008; Chan et al., 2009; Zhang et al., 2009a). *Tead1/Tead2*-null mice display a highly similar phenotype to that of *Yap1*-null mice providing additional support for this mechanism (Morin-Kensicki et al., 2006; Sawada et al., 2008).

Several studies using gene expression profiling have revealed a set of target genes that are induced by YAP (Dong et al., 2007; Zhao et al., 2007; Hao et al., 2008; Ota and Sasaki, 2008; Lu et al., 2010). Some of these genes, such as the IAP family member BIRC5 and the EGF family member amphiregulin (AREG), have been confirmed *in vitro* to mediate YAP-activated growth (Dong et al., 2007; Zhang et al., 2009b). However, the best-characterized direct target gene of YAP and TAZ is the connective tissue growth factor (CTGF). CTGF contains several TEAD-binding elements in its promoter region and was shown to mediate YAP-TAZ/TEAD-dependent proliferative and oncogenic functions. Microarray analysis identified an additional member of the CCN family, cystein-rich protein (Cyr61), as a target of YAP/TEAD (Zhao et al., 2007; 2008; Zhang et al., 2009a; Lai et al., 2011). Interestingly, CTGF and Cyr61 were found to be highly expressed in human cancers, where normal growth-restricting mechanisms are lost (Xie et al., 2001).

1.1.4 Upstream Regulators

Intense research over the past years established a relatively well-defined kinase cascade at the core of the Hippo pathway. However, the upstream signals, regulating this core kinase cascade, are not well defined and the subject of ongoing research. Most of what is currently known about the upstream signals was discovered in *Drosophila*. The first upstream regulators to be identified were the FERM domain containing apical cytoskeleton-binding proteins Merlin (Mer) and Expanded (Ex) (Hamaratoglu et al., 2006; Pellock et al., 2007). The deletion of both Mer and Ex led to tissue overgrowth phenotypes, similar to those observed by the deletion of core kinase components Hpo, Sav and Wts. Consistent with this observation, coexpression of Mer and Ex led to an activation of the pathway through an increase in Wts phosphorylation (Hamaratoglu et al., 2006). The protein Kibra was later shown to also interact with Mer and Ex, resulting in activation of the pathway (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). A milestone was the identification of the atypical cadherin Fat as the first transmembrane receptor protein signaling to the Hippo pathway (Bennett and Harvey, 2006; Cho et al., 2006; Hariharan, 2006; Silva et al., 2006; Willecke et al., 2006; Tyler and Baker, 2007). Fat functions by interacting with another atypical cadherin, Dachsous (Ds), on the adjacent cell. Other proteins shown to regulate Fat activity are the casein kinase Disc overgrown (Dco), the Golgi-resident kinase Four-jointed

(Fj) and the Fat/Ds-interacting protein Lowfat (Lft) (Matakatsu and Blair, 2006; Feng and Irvine, 2007; Ishikawa et al., 2008; Rogulja et al., 2008; Willecke et al., 2008; Mao et al., 2009; Sopko et al., 2009; Simon et al., 2010). Fat regulates the Hippo pathway by influencing expression and localization of Wts and Ex through mechanisms that involve the unconventional myosin Dachs (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006; Feng and Irvine, 2007; Tyler and Baker, 2007). In the search for additional cell surface proteins regulating the Hippo pathway, the apical transmembrane protein Crumbs (Crb) was recently discovered. This protein is important for the regulation of apical-basal cell polarity (Chen et al., 2010; Grzeschik et al., 2010; Robinson et al., 2010).

Even though mammalian homologs exist for most of the upstream components described for *Drosophila*, the significance and relevance of these proteins in regulating the activity of YAP *in vivo* in mammals remains poorly understood. An exception is the Neurofibromatosis 2 gene product (NF2), the Mer homolog. The deletion of NF2 *in vivo* in the murine liver leads to tumor formation, while additional deletion of one *Yap1* allele largely rescues the overgrowth phenotype. This suggests that both genes reside within the same pathway (reviewed in McClatchey and Giovannini, 2005; Zhang et al., 2010a).

1.1.5 The Physiological Role of Mammalian Hippo Signaling

Progress has been made in the quest to understand the physiological role of the Hippo signaling pathway components in mammals. Identifying the essential function of YAP in cell proliferation came from a study showing increased YAP expression levels in embryonic stem cells (Ramalho-Santos et al., 2002). It was later shown that deletion of *Yap1* in mice led to developmental defects that resulted in embryonic lethality at E8.5 (Morin-Kensicki et al., 2006). The phenotype for *Taz* (also called Wwtr1)-null mice is less drastic with embryos being viable at birth (Hossain et al., 2007; Tian et al., 2007; Makita et al., 2008). However, when *Yap1* and *Taz* are both deleted, anomalies occur during pre-implantation development and embryos die before the morula stage (Nishioka et al., 2009).

Great efforts have been made to elucidate the role of mammalian Hippo signaling in organ size regulation of the liver, the body's organ with the highest regeneration potential. In mice,

over a four-fold increase in liver size (hepatomegaly) was observed when the constitutively active YAP1-S127A was inducibly expressed. Remarkably, after ending the ectopic expression, the liver reversed to its original size (Camargo et al., 2007; Dong et al., 2007). Hepatomegaly also occurred when *Mst1/2* or *Sav1* were lost (Zhou et al., 2009; Lu et al., 2010; Song et al., 2010). However, it remains uncertain to what extent the discovered functional relationships of the Hippo signaling transduction in the liver are conserved and active in other tissues (Zhou et al., 2009). Stem cells are essential for organs to reach their final size. Interestingly, YAP expression in the intestine and in the epidermis was found to be restricted to the compartment that comprises the tissue resident stem cells (Camargo et al., 2007).

1.2 The Skin

The skin is the largest organ in mammals, covering the entire body surface (in humans an average of 1.5-2 m^2) and forming a barrier between the organism and its surrounding environment. One of its various functions is to protect the organism against environmental stresses, such as pathogens, UV radiation, chemical and mechanical stress. Other functions are the regulation of hydration and temperature of the body (Chuong et al., 2002; Blanpain and Fuchs, 2006; Segre, 2006). The skin serves an important role beyond the barrier-function. It provides a tactile sense, which enables the animal to obtain information about the environment. Additionally, it is important for social interactions between animals, as a form of communication via pigment pattern, hair and fur (Blanpain and Fuchs, 2006). Moreover, it plays an important role for immunologic, endocrine and metabolic processes (Chuong et al., 2002). Many appendages are formed in the mammalian skin, which include hair follicles, nails, sebaceous (oil) and sweat glands, all of which are crucial for its functions.

1.2.1 Structure of the Skin

In mammals the skin comprises the epidermis, the dermis and the subcutis (or hypodermis). Figure 3 shows the structure of the skin. The innermost layer of the skin, the subcutis, consists

of adipocytes and lies below the dermis (Montagna and Parakkal, 1974; Alberts et al., 2008). Right above the subcutis lies the dermis, a mesenchymal connective tissue. The dermis is composed of fibroblasts and extracellular matrix (ECM) components (e.g. collagen and elastic fibers). The subcutis and dermis contain many blood vessels and nerves, and hence, provide not only stability but also nutrients for the outermost layer of the skin, the epidermis. The cross talk between dermis and epidermis is critical for proliferation and morphogenesis of the epidermal compartment (Sengel, 1986; Chuong, 1998). The epidermis is a multilayered (stratified) and highly specialized epithelium that is separated from the underlying dermis by the basement membrane (BM). It comprises the interfollicular epidermis (IFE), which describes the epidermis in between the hair follicles. The IFE contains as associated appendages hair follicles (HFs), sebaceous glands (SGs) as well as sweat glands (Figure 3).

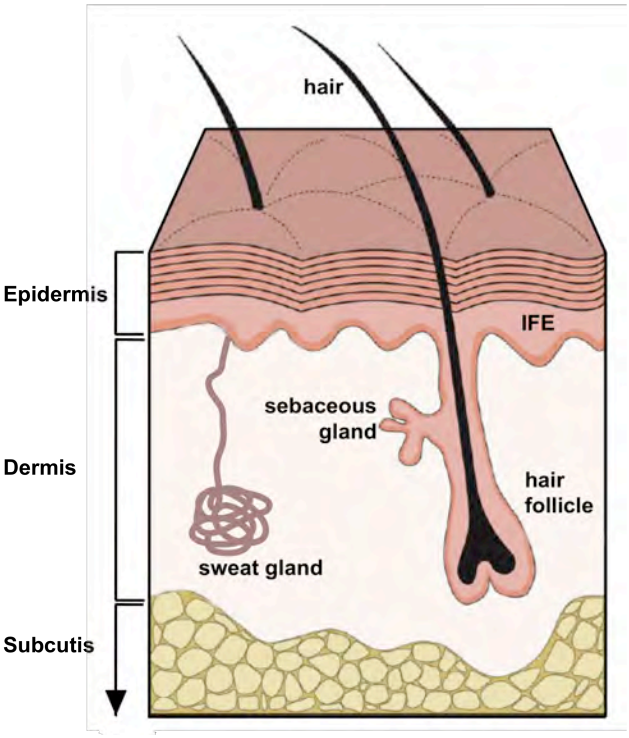

Figure 3: Structure of the Skin.

The skin consists of the epidermis, dermis and subcutis. Appendages of the interfollicular epidermis (IFE) are hair follicles, sebaceous glands and sweat glands. Modified after Aasen et al. 2010.

The basal layer of the IFE contains the interfollicular proliferative cells, which eventually withdraw from the cell cycle and commit to terminal differentiation (Koster and Roop, 2007). In the process of building the multilayered epidermis (Figure 4), the basal cells detach from the BM and move upwards, through different stages of differentiation (Fuchs, 2008). The first

suprabasal differentiated layer is the spinous layer, containing transcriptionally active cells that produce keratin filaments connected to desmosomes, laying the groundwork for the mechanical infrastructure of the suprabasal epidermis. The process of the basal-spinous transition is regulated by various signals, for example the switch from keratin K14/K5 expression to keratin K1/K10 expression (Fuchs and Green, 1980; Stoler et al., 1988; Yuspa et al., 1989; Bickenbach et al., 1995). Right above the spinous layer follows the granular layer. The cells of the granular layer produce lipid-rich lamellar granules and contribute to establishing the cornified barrier function of the epidermis. Finally, the outermost protective layer of the epidermis, the stratum corneum, consists of enucleated, dead cells that are ready to be shed (Koster and Roop, 2007; Fuchs, 2008).

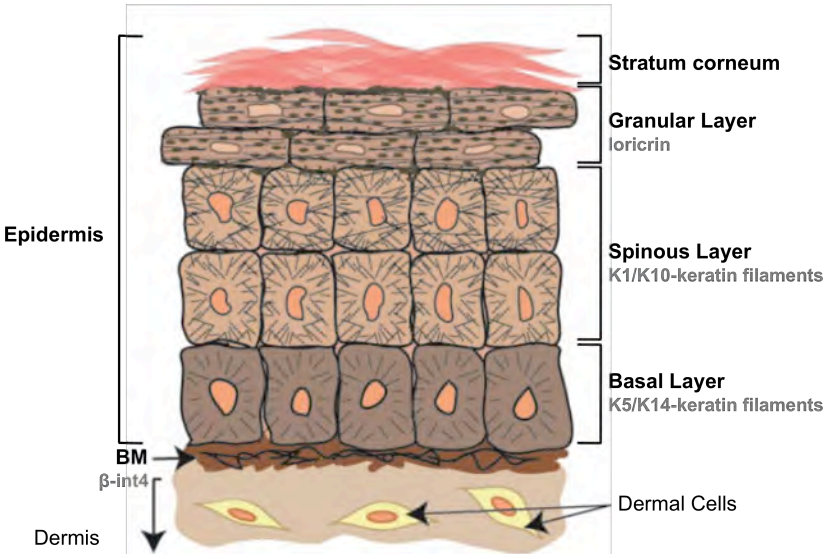

Figure 4: Structure of the Epidermis.

Schematic showing the different layers of the epidermis. The epidermis is separated from the dermis by the basement membrane (BM). The basal layer comprises the progenitor cells of the epidermis and expresses keratin 5 (K5) and keratin 14 (K14). The basal cells eventually withdraw from the cell cycle and enter a differentiation program, detaching from the BM and moving upwards. The BM expresses integrin-β4 (β-int4), a component of hemidesmosomes. The first differentiated layer above the basal layer is the spinous layer, followed by the granular layer. The spinous layer expresses keratin 1 (K1) and keratin 10 (K10), while the granular layer expresses loricrin. Modified from Alonso and Fuchs 2003.

One of the epidermal appendages is the hair follicle (HF), which is composed of the outer root sheath (ORS), companion layer, inner root sheath (IRS), hair shaft and the rapidly proliferating matrix cells in the hair bulb, which give rise to the different hair lineages (Niemann and Watt, 2002; Blanpain and Fuchs, 2006; Shimomura and Christiano, 2010). The best-characterized multipotent hair follicle stem cells have been shown to reside within the permanent part of the HF, the so-called bulge (Cotsarelis et al., 1990; Nowak et al., 2008). Whereas under homeostatic conditions regeneration of the IFE is a continuous process, the hair follicle undergoes cycles of growth (anagen), apoptosis-mediated regression (catagen), and quiescence (telogen) (Alonso and Fuchs, 2006).

Another epidermal appendage is the sebaceous gland (SG), which is attached to the HF. The SG contains an undifferentiated layer of keratinocytes at the periphery of the gland and terminally differentiated sebocytes in the center. Mature sebocytes are lipid-filled and eventually burst, releasing their contents onto the surface of the skin (Niemann, 2009; Schneider et al., 2009).

1.2.2 Epidermal Stem Cells

Similar to the gastrointestinal and hematopoietic systems, the epidermis is a rapidly renewing tissue, which makes it ideal for studying stem cell biology. Characteristics by which stem cells (SCs) are being identified are their capability of unlimited or prolonged self-renewal, their proliferation potential, their long-term repopulation capacity as well as their potential to generate at least one type of highly differentiated cell type (Lajtha, 1979; Hall and Watt, 1989; Morrison et al., 1997; Potten, 1997; He et al., 2009).

Epidermal SCs have been shown to be multipotent, and are needed for building the epidermis during development (Watt, 1998). One single adult skin SC has the proliferative capacity to produce epidermis covering the complete body surface (Rochat et al., 1994). But SCs are also important in adult life, for the process of continual and balanced cell replacement, called tissue homeostasis, which is critical for maintaining an intact adult epidermis. Human epidermis is thicker compared to murine epidermis and has a frequent cell turn-over, self-renewing within four weeks (Blanpain et al., 2007; Pincelli and Marconi, 2010). In mice, the

proliferation rate slows down in the postnatal epidermis as the hair coat develops and takes over most of the protective functions (Fuchs, 2008). In the epidermis stem cells have been found to reside in the bulge area of the HF, the IFE and the SG (Figure 5).

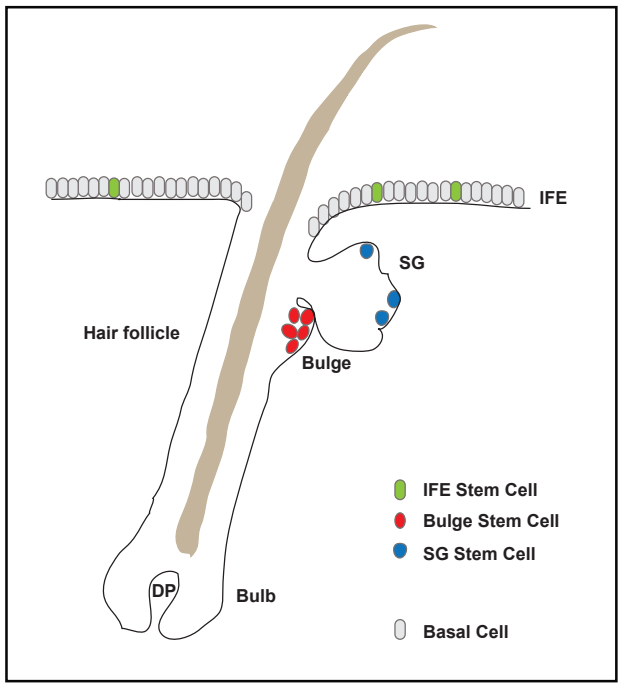

Figure 5: Stem Cells in the Epidermis.

Stem cells in the epidermis are located in the interfollicular epidermis (IFE), the sebaceous gland (SG) and the bulge region of the hair follicle (HF). The bulb of the hair follicle is in contact with the dermal papilla (DP).

1.2.2.1 Heterogeneity of Epidermal Stem Cells

In the quest for the identification of epidermal stem cells, lineage-tracing and label-retaining studies utilizing the characteristic of these cells to be slow-cycling brought some important answers. By injecting nucleotide analogs (BrdU or ^{3}H-thymidine) at a time when the epidermis is actively proliferating and thus incorporating the label, it was now possible to trace the cells that were retaining the label over a long time period, which were considered to be the stem cells (label-retaining cells, LRCs) (Potten, 1974; Bickenbach, 1981; Morris et al., 1985; Potten and Morris, 1988; Braun and Watt, 2004; Fuchs, 2009). It became clear in these label-retaining studies that the epidermis contains at least two distinct LRC populations with a different cell cycle duration and moreover a non-label-retaining, post-mitotic population.

Accordingly, the most widely accepted model for the explanation of cell replacement in the epidermis is that of the epidermal proliferative unit (EPU), which implies proliferative heterogeneity (Mackenzie, 1969; 1970; Allen and Potten, 1974; Potten, 1974). Each EPU contains in its center of approximately 10 to 11 basal cells one slow-cycling and thus long-term label-retaining cell (SC), which gives rise to SC daughters, as well as rapidly proliferating daughter cells which are termed transit amplifying (TA) cells (Bickenbach, 1981; Potten, 1981; Cotsarelis et al., 1989; Pincelli and Marconi, 2010). Furthermore the EPU contains three flattened suprabasal cells and five to seven cornified cells, making a total of 18 to 21 cells per EPU (Potten, 1974; Mackenzie, 1975; Ghazizadeh and Taichman, 2001).

The finding, that infrequently dividing epidermal SCs produce frequently proliferating TA daughters that eventually start to differentiate after a limited number of cell divisions, has been supported by studies analyzing the cell kinetics in the epidermis (Potten, 1974; Jones et al., 1995; Fuchs and Horsley, 2008). It has been shown that in the epidermis cell division is restricted to the cells in the basal layer, but there are observations that not all of these cells capable of dividing are in fact stem cells (Morris et al., 1985). About 10% of the basal cells are assumed to be stem cells, which has been concluded from a study in which murine epidermis had been severely damaged by radiation and the number of basal cells with a clonogenic potential to renew the epidermis was quantified (Withers, 1967; Potten and Hendry, 1973). This leaves an additional 50% of dividing basal cells, which might represent the TA population, whereas post-mitotic cells make up the remaining 40% of basal cells (Iversen et al., 1968; Potten and Morris, 1988). A similar percentage of stem cells in murine epidermis was also confirmed with *in vitro* studies looking at the colony-forming efficiency and showed that the frequency of clonogenic keratinocytes in culture lies between 2% and 8% (Morris et al., 1988; Bickenbach and Chism, 1998).

The hierarchy of epidermal stem cells has also been supported by clonogenic assays with human epidermal cells, where *in vitro* replating-experiments have identified three types of keratinocyte clones: holoclones (28%), paraclones (49%) and meroclones (23%) (Barrandon and Green, 1987). Holoclones have the highest proliferation potential in long-term culture, give rise to the largest colonies and are thought to arise from stem cells. Paraclones have a more limited growth potential (approximately 15 cell generations), form smaller colonies that

ultimately differentiate and are thus assumed to represent the TA cells. Meroclones are an intermediate between holoclones and paraclones in terms of their appearance and their reproductive behavior (Barrandon and Green, 1987).

Recently the EPU model with its stem cell heterogeneity has been questioned by lineage-tracing experiments showing that adult epidermis is instead maintained by a single population of progenitors (Clayton et al., 2007). It has to be taken into account, however, that this study was contemplated in murine tail epidermis, whereas most previous studies have been done either in murine back epidermis or cultivated primary human keratinocytes. Regional as well as age-related differences might be reflected in the varying results. The tail epidermis, for example, not only contains fewer HFs but is also organized in squames or scales, and thus it cannot be ruled out that there might be a different underlying system of stem cell proliferation in this particular region.

1.2.2.2 Independent Stem Cell Populations in Hair Follicle and Interfollicular Epidermis

Already in the 1960s, it could be demonstrated that the HF completely regenerated after surgical removal of the bulb region of the HF, indicating that the SCs are not located in this region (Oliver, 1966a; 1966b). However, the hair bulb does contain the so-called matrix cells, which are highly proliferative cells (TA cells) that give rise to the hair and IRS. Label-retaining studies in the murine HF made it clear that the SCs of the HF, which are responsible for the cyclic regeneration of the HF, are located in the bulge region (Figure 5 ; Cotsarelis et al., 1990; Rochat et al., 1994; Oshima et al., 2001). It was calculated from subsequent LRC studies that approximately 95% of the slow-cycling epidermal LRCs reside in the bulge (Braun et al., 2003; Blanpain et al., 2004; Tumbar, 2004). Moreover, cells isolated from the bulge of rat as well as human HFs by microdissection showed the highest colony-forming efficiency (Rochat et al., 1994; Oshima et al., 2001).

It was assumed for a while that the SCs in the HF bulge constitute the true SCs of the epidermis, giving rise to all the different lineages of the epidermis. And while indeed it is confirmed that HF SCs can regenerate the complete epidermis with all its lineages upon

injury, it has also been demonstrated that under homeostatic conditions the HF SCs do not contribute to the maintenance of the IFE (Braun et al., 2003; Tumbar, 2004; Levy et al., 2007; Langton et al., 2008). Additionally, evidence for a long-term populating interfollicular SC came from *in vivo* lineage-tracing studies (Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002; Schneider et al., 2003; Ghazizadeh and Taichman, 2005; Ito et al., 2005; Langton et al., 2008). An interesting study demonstrates that upon loss of HFs, the survival of the adjacent IFE was not affected (Ito et al., 2005). These studies provide strong evidence for an independent SC population in the IFE, albeit this population is not well characterized to date.

1.2.3 Cell Adhesion in the Skin

The formation of a multi-cellular organism of higher complexity, with various tissues and organs, requires the interaction of different cell types. A close interaction between adjacent cells through intercellular junctions is a pre-requisite for achieving this goal. Intercellular junctions play a crucial role in epithelial tissues such as the skin, since they provide the necessary stability that is needed for tissue morphogenesis and also for body movement. They are essential for the proper physiology and barrier function of the skin (Niessen, 2007). The epidermis is a dynamic tissue with its cells moving upwards in their differentiation process during homeostatic self-renewal. And also during development, organ growth, and wound repair, adhesive contacts need to get remodeled. Therefore, intercellular junctions face a special challenge. They need to ensure the adhesive properties necessary for epithelial sheet and barrier formation, but at the same time they need to be adaptive and ready to rearrange to allow for the dynamic changes in cell adhesion (van Roy and Berx, 2008).

1.2.3.1 Adherens Junctions

Three of the junctional complexes that are important for proper epithelial sheet formation are tight junctions (TJs), adherens junctions (AJs) and desmosomes (Matter and Balda, 2003; Perez-Moreno et al., 2003; Yin and Green, 2004). Together they form the apical junctional complex (Figure 6), which regulates cell polarity, permeability and adhesion (Farquhar and Palade, 1963).

Most apically located within the vertebrate cell are the TJs (zona occludens), which serve two main functions. They seal neighboring cells closely together and function as a selective permeability barrier (limiting free diffusion of molecules). In addition they also ensure the separation of apical and basolateral membrane components, which is their second so-called "fence function" (D'Atri and Citi, 2002; Tsukita and Furuse, 2002). Desmosomes play, similar to AJs, an important role for intercellular adhesion, but they are located basolateral within the cell and provide anchorage sites for intermediate filaments, which belong to the keratin family in most epithelial cells (Garrod et al., 2002; Huber, 2003; Garrod and Chidgey, 2008).

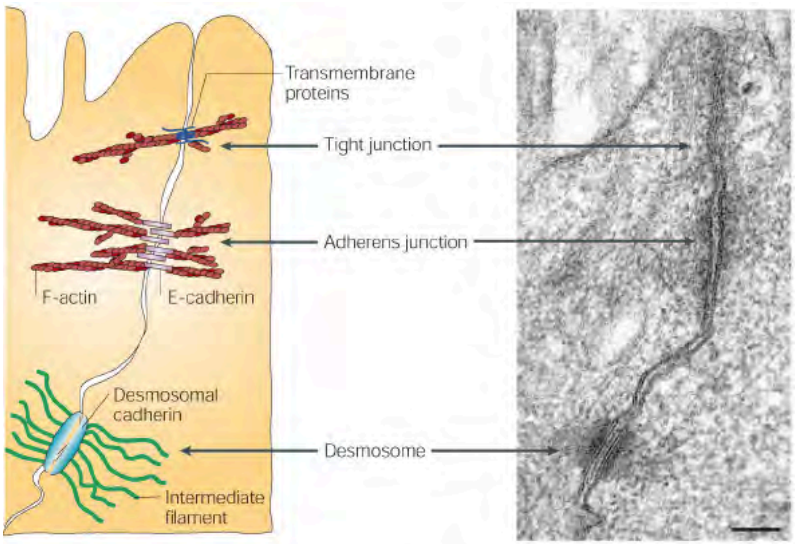

Figure 6: Intercellular Junctions.

Tight junctions (TJs), adherens junctions (AJs) and desmosomes form the apical junctional complex. Figure from Kobielak and Fuchs 2004.

AJs are positioned directly below the TJs and are as their name already suggests crucial for cell-cell adhesion (Tepass, 2002; Perez-Moreno et al., 2003). In epithelial tissues, such as the skin, AJs provide a connection to the cytoskeleton of the cell, forming a continuous adhesion belt. Lately functions for AJs have been identified that go beyond their mere role in securing tissue integrity. A view of AJs as a hub for signaling molecules and polarity cues is emerging, which make them a likely candidate for sensing and relaying information during tissue growth (Nelson and Nusse, 2004; Perez-Moreno and Fuchs, 2006). Indeed, there is evidence emerging that AJs play a role in restricting basal cell proliferation and in sensing epidermal cell density (Lien et al., 2006a).

The core components of the AJ complex are the cadherin adhesion molecules (Takeichi, 1990). In epithelial tissues E(epithelial)-cadherin is prevalent, which like all cadherins, contains two or more extracellular cadherin domains that form a homotypical Ca^{2+}-dependent interaction with the E-cadherins of the neighboring cell (Leckband and Prakasam, 2006; Pokutta and Weis, 2007). While their extracellular domains ensure the adhesion to the adjacent cell, their conserved cytoplasmic tail interacts with a set of cytoplasmic proteins, the catenins, β-catenin, p120-catenin, and α-catenin, that build the bridge to the actin cytoskeleton (Perez-Moreno and Fuchs, 2006; Pokutta and Weis, 2007; Nishimura and Takeichi, 2009).

1.2.3.2 Key Cytoplasmic Regulators at the Adherens Junctions

The catenins, which form the connection to the cytoplasmic E-cadherin domains, are very important for the function of the AJs since they are ensuring a stable intercellular adhesion by providing the link to the actin cytoskeleton. While this structural role of the catenins is well established, recent studies have shown that they also serve an additional function as signaling molecules (Perez-Moreno and Fuchs, 2006). The best-studied example is β-catenin, with its dual role in cell adhesion and as a transcriptional coactivator in the Wnt signaling pathway (Nelson and Nusse, 2004; Bienz, 2005). But also for p120 catenin and α-catenin studies are revealing additional roles in cellular processes that go beyond cell-adhesion (Vasioukhin et al., 2001; Reynolds and Roczniak-Ferguson, 2004). Another component of the AJs is plakoglobin (also called γ-catenin).

While p120-catenin, β-catenin and plakoglobin belong to the armadillo family of proteins and share sequence similarity, α -catenin differs notably in both sequence and structural organization. p120-catenin, β-catenin and plakoglobin bind directly to the cytoplasmic domain of E-cadherin, whereas α -catenin joins the complex via binding to β -catenin or plakoglobin and providing the link to the actin cytoskeleton (Figure 7). The binding of α-catenin to β-catenin and actin has been shown to be mutually exclusive (Drees et al., 2005; Yamada et al., 2005).

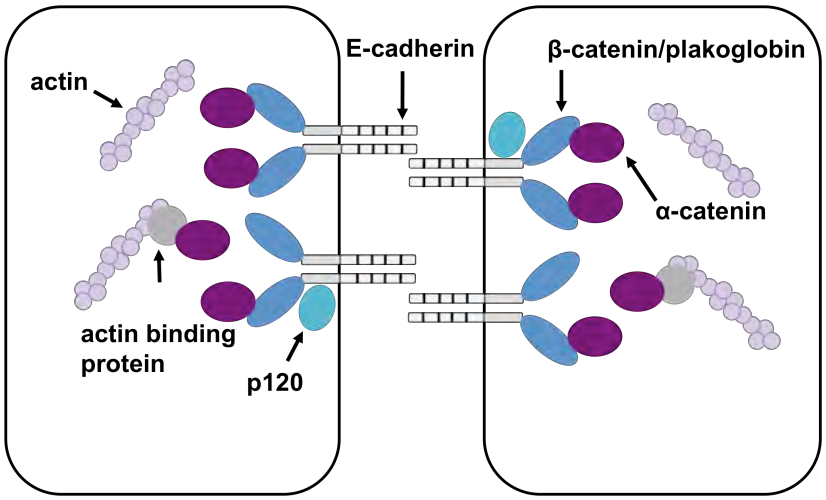

Figure 7: Structure of the Adherens Junctions.

p120-catenin and β-catenin/plakoglobin directly bind to the cytoplasmic domain of E-cadherin. α-catenin binds to β-catenin or plakoglobin and provides the link to the actin cytoskeleton through different actin binding proteins.

1.2.3.3 α-Catenin's Function in Cell Proliferation and Carcinogenesis

The α-catenin protein family contains three different forms. The founding member α-E-catenin (hereafter α-catenin) is expressed in epithelial tissues such as the skin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). The other forms are α-N-catenin, which is restricted to neural tissues and α-T-catenin, which is predominantly found in the testis and the heart.

Genetic studies in mice have demonstrated the requirement for α-catenin during embryogenesis at the blastocyst stage. Loss of α-catenin results in an embryonic lethal phenotype, similar to that observed upon E-cadherin loss (Larue et al., 1994; Kofron et al., 1997; Torres et al., 1997). Moreover, it was shown that α-catenin is essential for the proper function of AJs in epithelial cells *in vitro* and *in vivo* and in fact serves an important function in epidermal biology (Vasioukhin et al., 2000; 2001). Whereas β-catenin has long been acknowledged to play dual roles in cell adhesion and as a signaling molecule, the same has only recently become recognized for α-catenin as well (Nelson and Nusse, 2004). The conditional deletion of α-catenin in the developing epidermis on embryonic day 14.5 led to an impairment in hair follicle development and epidermal morphogenesis (Vasioukhin et al.,

2001). Intercellular adhesion through AJs was compromised, even though the AJ components E-cadherin and β-catenin were still localizing correctly to the cell membrane. Surprisingly, the conditional loss of α-catenin in the epidermis also led to hyperproliferation in the epidermis. When plated *in vitro*, α-catenin-null keratinocytes were poorly contact inhibited and showed a high proliferation rate (Vasioukhin et al., 2001). These results suggest that α-catenin regulates stem cell proliferation, and demonstrate an inverse correlation of adhesion and proliferation.

The implication for AJs in proliferative processes is further supported by observations showing that mutations in AJ-proteins not only result in tissue degeneration, but they also lead to tumor formation and metastasis, processes where proliferation has gone awry (Yagi and Takeichi, 2000; Tepass, 2002; Perez-Moreno et al., 2003). It is known by now that a reduction of α-catenin and also E-cadherin levels in a variety of tissues contributes to tumor formation in humans (Kadowaki et al., 1994; Ochiai et al., 1994; Shiozaki et al., 1994; Ewing et al., 1995; Rimm et al., 1995; Kozyraki et al., 1996). Null mutations of α-catenin are also frequently found in epithelial cancers, where they correlate more highly with the degree of tumor invasiveness than those of E-cadherin (Shimoyama et al., 1992; Morton et al., 1993; Kadowaki et al., 1994; Matsui et al., 1994; Ewing et al., 1995; Bullions et al., 1997). The function of α-catenin as a tumor suppressor was moreover demonstrated in the skin by a transplantation experiment, showing that the grafting of α-catenin-null skin onto Nude mice led to the formation of squamous cell carcinoma (SCC) (Kobielak and Fuchs, 2006). The results obtained upon α-catenin ablation are making it necessary to reconsider the existing view that defects in intercellular adhesion are occurring later in the process of carcinogenesis, and instead suggest that they are earlier steps than previously assumed. The mechanisms how exactly α-catenin mediates these processes are unclear.

1.2.4 Hippo Signaling in the Skin

The epidermis is a rapidly renewing tissue. Epidermal stem cells are essential for both skin homeostasis and regeneration during wound repair. Aberrant proliferation in the epidermis leads to skin diseases such as psoriasis and epidermal cancer formation. Therefore, it is important to understand the mechanisms that control proliferation in the epidermis. The Hippo signaling pathway has been shown to be involved in the regulation of epidermal proliferation by one previous study. In this study, loss of *Sav1* (WW45), a core component of the Hippo cascade, in the epidermis induced an expansion of the basal progenitor cell population (Lee et al., 2008). Moreover, the phosphorylation of YAP, the regulatory downstream event rendering YAP inactive in the cytosol, could no longer be observed *in vitro* in cultured *Sav1*-null keratinocytes, implying an effect on YAP activity (Lee et al., 2008). Interestingly, YAP has been shown to be expressed in the basal layer of the epidermis, the compartment that comprises the tissue resident stem and progenitor cells (Camargo et al., 2007). Future research will have to elucidate how the Hippo signaling pathway controls tissue homeostasis and tissue growth in the epidermis.

2. Aims

Research in recent years has begun to discover a role for the Hippo signaling pathway in the regulation of organ size not only in *Drosophila* but also in mammals. In the murine liver, constitutive activation of the pathway's downstream effector, YAP, caused a more than four-fold increase in liver size (Camargo et al., 2007; Dong et al., 2007). However, little is known about the endogenous mechanisms that provide information about organ size and relay this information to the organ's resident stem cells.

Interestingly, YAP has been found to be expressed in stem cell compartments of the intestine and also of the epidermis (Camargo et al., 2007). Moreover, the deletion of one of the core cascade components, *Sav1*, resulted in an expansion of progenitor cells in the epidermis (Lee et al., 2008). Based on these results, the aim of this thesis was to further investigate the function of Hippo signaling in the epidermis, with a focus on YAP. Understanding the function of this novel pathway in the epidermis could prove therapeutically relevant for developing treatments for skin diseases associated with aberrant proliferation (e.g. psoriasis) as well as skin cancers. Moreover, it could ultimately lead to an advancement of current skin transplantation therapies.

Aim 1:

Investigate the Effect of *Yap1* Activation in the Epidermis

The deletion of core proteins of the Hippo pathway in *Drosophila* produces phenotypes similar to that obtained upon Yki (YAP homolog)-overexpression. In the liver activation of *Yap1* led to an increase in liver size (Camargo et al., 2007; Dong et al., 2007). Based on this finding, combined with the published results obtained for *Sav1* loss in the epidermis, we hypothesize that expression of mutated, constitutively active form of YAP (YAP1-S127A), which is predominantly nuclear, leads to an expansion of epidermal progenitors. To test this, we will utilize transgenic mice carrying a doxycycline (Dox)-inducible allele of the mutated *Yap1-S127A* . We plan to use a genetic strategy to limit YAP1-S127A expression to the basal layer of the epidermis, utilizing the Cre-mediated activation of a reverse tetracycline-

dependent transactivator (rtTA) selectively in the progeny of K14-expressing cells of stratified epithelia (Dassule et al., 2000; Belteki et al., 2005). Mice carrying these transgenes will be referred to as Tg in this work.

Aim 2:

Investigate the Effect of *Yap1* Deletion in the Epidermis

The systemic deletion of *Yap1* in the mouse is lethal at embryonic day E8.5, therefore preventing the examination of *Yap1* loss in the epidermis. To circumvent this problem, we generated a mouse with a conditional *Yap1* allele, where exon 1 and 2 were flanked by loxP sites, allowing tissue-specific excision upon Cre-expression. We plan to delete *Yap1* by expressing Cre under the K14-promoter, and to study the potential effect on epidermal biology. K14 expression is restricted to the basal layer in the epidermis, known to comprise the epidermal progenitor cells.

Aim 3:

Investigate the Function of the TEAD Transcription Factors in Epidermal Biology

It is known that the serine residue S94 in human YAP is crucial for the interaction with the transcription factor TEAD, which is thought to mediate most of the Hippo pathway dependent signals. In one study, the pro-proliferate functions of YAP were greatly diminished when S94 was mutated to alanine (S94A), thus impairing the interaction of YAP and TEAD (Zhao et al., 2008). However, YAP has been demonstrated to bind to other transcription factors as well, and a tissue-dependent effect cannot be ruled out (Yagi et al., 1999; Strano et al., 2001; Vassilev et al., 2001; Ferrigno et al., 2002; Zaidi et al., 2004). We thus wanted to evaluate the function of the TEAD transcription factors in Hippo signaling, specifically in epidermal biology. To address this question *in vivo*, we generated a *Yap1* mutant allele containing the S79A mutation, which is the murine equivalent to S94A in human YAP. We plan to study the effect of this allele in the epidermis by combining it with the *Yap*1 conditional loss-of-function allele (Aim 2), which will be deleted by Cre expressed under the K14-promoter, leaving YAP1-S79A as the remaining functional protein.

Aim 4:

Investigate the Function of the MST1/2 Kinases in Epidermal Biology

Deletion of both *Mst1* and *Mst2* in the liver results in YAP activation and hepatomegaly, suggesting a function for these kinases as negative regulators *in vivo* (Zhou et al., 2009; Song et al., 2010). We wanted to assess whether deletion of both *Mst1* and *Mst2* in the skin would lead to YAP activation and produce a similar phenotype of tissue overgrowth. We plan to study mice generated in the laboratory of Joseph Avruch (MGH), carrying the $Mst1^{-/-}$ $Mst2^{-/fl}$ allele by crossing them with mice expressing K14-Cre to ablate MST1/2 expression in the basal cells of the epidermis. We plan to complement the *in vivo* studies with *in vitro* experiments in the keratinocyte cell line HaCaT, using siRNAs to knock down the transcript of *Mst1* and *Mst2*. These results will provide valuable information about the conservation of the Hippo core kinase cascade across different tissues of the body.

3. MANUSCRIPT I

Yap1 Acts Downstream of α -Catenin to Control Epidermal Proliferation

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Experimental Contribution

The part of my work presented in the publication "Yap1 Acts Downstream of α-Catenin to Control Epidermal Proliferation" was the *in vivo* characterization of the role of the Hippo signaling pathway in the epidermis, utilizing transgenic mouse models for gain- and loss-of-function of *Yap1*, the mutated *Yap1*-S79A and *Mst1/2* loss-of-function. The *Yap1* cKO and *Yap1*-S79A mice were generated during my diploma thesis work. The initial ideas and the conceptual design of my work were developed together with my advisor, Assistant Professor Fernando Camargo. Experiments were planned, performed and evaluated by myself. I wrote the manuscript together with Fernando Camargo and Morvarid Mohseni.

The following figures present my work:
Figure 1 A, C-G; Figure 2 A-E; Figure 3 A-G; Figure 4 D-E; Figure 7 C
Figure S1 A-H; Figure S2 A-C; Figure S3 A-C; Figure S4 A-C, E

The original article including the supplemental information is included on the following pages and online available at:
http://www.cell.com/abstract/S0092-8674(11)00183-8

Cell

Yap1 Acts Downstream of α-Catenin to Control Epidermal Proliferation

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SUMMARY

During development and regeneration, proliferation of tissue-specific stem cells is tightly controlled to produce organs of a predetermined size. The molecular determinants of this process remain poorly understood. Here, we investigate the function of Yap1, the transcriptional effector of the Hippo signaling pathway, in skin biology. Using gain- and loss-of-function studies, we show that Yap1 is a critical modulator of epidermal stem cell proliferation and tissue expansion. Yap1 mediates this effect through interaction with TEAD transcription factors. Additionally, our studies reveal that α-catenin, a molecule previously implicated in tumor suppression and cell density sensing in the skin, is an upstream negative regulator of Yap1. α-catenin controls Yap1 activity and phosphorylation by modulating its interaction with 14-3-3 and the PP2A phosphatase. Together, these data identify Yap1 as a determinant of the proliferative capacity of epidermal stem cells and as an important effector of a "crowd control" molecular circuitry in mammalian skin.

INTRODUCTION

During mammalian development, proliferation and cell death of tissue-specific progenitor and stem cells (SCs) need to be tightly monitored and controlled to produce organs of a predetermined size. Experimental manipulation of SC numbers or activity during embryogenesis can thus have striking effects on the final size of certain organs (Depaepe et al., 2005; Kim et al., 2005; Stanger et al., 2007). Such exquisite regulatory mechanisms also orchestrate homeostasis of adult tissues and regenerative processes whereby the final shape and size of organs can be restored after cellular loss. Though a number of signaling molecules have been implicated in controlling SC proliferation, little is known about endogenous mechanisms that "sense" or provide information about organ size. It is also unclear how these mechanisms relay needs for tissue growth to its resident SCs and how these cues are translated into SC proliferation or apoptosis.

The Hippo signaling pathway was initially discovered in *Drosophila* as a potent mechanism that restricts tissue size by limiting cell proliferation and promoting apoptosis. At the core of this pathway is a kinase cascade composed of four tumor suppressors, including Hippo (Hpo) and Warts (Wts) and its regulatory proteins Salvador and Mats (Harvey et al., 2003; Lai et al., 2005; Pantalacci et al., 2003; Wu et al., 2003; Xu et al., 1995). The Hpo-Sav complex phosphorylates and activates the Wts-Mats complex, which in turn phosphorylates and inactivates the transcriptional coactivator Yorkie (Yki) (Huang et al., 2005; Oh and Irvine, 2008). Yki phosphorylation prevents its nuclear translocation (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007), wherein it acts as a coactivator for the TEAD/TEF family transcription factor Scalloped (Sd) (Wu et al., 2008; Zhang et al., 2008). This core set of Hippo signaling components is highly conserved in mammals, and orthologs of Hpo (Mst1/2), Sav (WW45), Wts (Lats1/2), and Yki (Yap1) exhibit similar biochemical properties in cultured cells (Dong et al., 2007; Oka et al., 2008; Zhao et al., 2007). The importance of Hippo signaling in mammalian organ size control has been studied extensively in the liver, where loss of Mst1/2 or Sav or overexpression of Yap1 lead to hepatomegaly (Camargo et al., 2007; Dong et al., 2007; Lu et al., 2010; Song et al., 2010; Zhou et al., 2009). However, to what extent these predicted epistatic and functional relationships are conserved and active in other tissues remains uncertain (Zhou et al., 2009).

Compared with the core kinase cascade regulating Yki phosphorylation, components acting upstream of this complex are less well defined. Earlier work in *Drosophila* has implicated the apical membrane-associated FERM-domain proteins Merlin (Mer), Expanded (Ex), and Kibra as pathway components upstream of Hpo (Baumgartner et al., 2010; Genevet et al., 2010; Hamaratoglu et al., 2006). Recent studies further implicated the apical transmembrane protein Crumbs (Crb) (Robinson et al., 2010) and the atypical cadherin Fat (Ft) (Hariharan,

2006) as modulators of the fly Hippo pathway. However, with the exception of the mammalian Merlin ortholog NF2 (Zhang et al., 2010), the significance and relevance of these proteins in regulating the activity of Yap1 in vivo in mammals are largely unclear. The identification of physiological upstream regulators of mammalian Hippo signaling could provide important insights into the mechanisms sensing and controlling organ size.

The mammalian epidermis is a rapidly regenerating epithelial tissue whose maintenance depends on the self-renewing ability of epidermal SCs residing in the basal layer. After division, short-lived progenitor cells leave the basal layer and move through the suprabasal layers to the tissue surface as they terminally differentiate (Fuchs, 2007). Epidermal growth must be carefully balanced: inadequate proliferation results in thinning of the skin and loss of protection, and excessive growth is characteristic of hyperproliferative disorders. During development, the epidermis must be able to sense needs for expansion and replace basal vacancies, and during regeneration, cells must migrate and proliferate but also sense when to stop after wound closure. It has been postulated that adherens junctions (AJs), cadherin and catenin-based cell adhesion structures, might have a function in sensing epidermal cell density and restricting basal cell proliferation (Lien et al., 2006b). For instance, loss of some, but not all, AJ components, such as α-catenin or p120-catenin, triggers severe epidermal hyperproliferation and tumors in transgenic mice (Kobielak and Fuchs, 2006; Perez-Moreno et al., 2006; Vasioukhin et al., 2001). The mechanisms acting downstream of these complexes are not fully understood. Intriguingly, it has been recently found that NF2 can modulate epidermal development via an association with AJs, specifically α-catenin, and the Par3 polarity complex (Gladden et al., 2010). These findings raise the possibility that signaling cues that regulate cell density and cell polarity could converge on the regulation of downstream Hippo signaling components.

Here, we utilize gain- and loss-of-function models in mice to show that Yap1 is an essential regulator of epidermal maintenance and SC proliferative capacity. In addition, we identify α-catenin, an AJ component and a known tumor suppressor in the skin, as a crucial upstream negative regulator of Yap1 in epidermal cells in vitro and in vivo. Our work identifies a direct link between a signaling component of cell density-dependent AJs and a transcriptional regulator and provides a paradigm for the regulation of tissue expansion in response to extracellular cues.

RESULTS

Activation of Yap1 Can Expand the Epidermal SC Compartment

In newborn mouse epidermis, Yap1 has a dynamic subcellular localization pattern in the basal compartment where it is present in the nucleus in some cells and in the cytoplasm/membrane of others. Expression of Yap1 is reduced and in a more diffuse nonnuclear pattern in the suprabasal-differentiated cell layers (Figure 1A). This pattern is also observed in cultured human keratinocytes in which high cellular densities lead to Yap1 relocalization out of the nucleus (Figure 1B). Thus, regulation of Yap1 subcellular localization might be important for the switch between proliferative and terminally differentiating compartments. To test this, we utilized transgenic mice carrying a doxycycline (Dox)-inducible allele of a mutated version of Yap1 (S127A). This mutation results in the enhanced nuclear localization of Yap1 (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007). To restrict Yap1 activation to the epidermis, we developed a binary genetic strategy based on the Cre-mediated activation of a reverse tetracycline-dependent transactivator selectively in the progeny of K14-expressing progenitors of stratified epithelia (Belteki et al., 2005; Dassule et al., 2000) (Figure S1A available online). Mice carrying these transgenes will be referred to as Tg hereafter. Eight days after Dox administration, adult Tg mice developed thickening and wrinkling of the skin. Histological analyses demonstrated that, contrary to the typical one-/two-cell layer common to adult mouse epidermis, Tg skin developed into a multilayered epithelium (Figure 1C). Staining for the proliferation marker Ki-67 revealed a substantial increase in the number of proliferating basal cells and an extension of the proliferative domain into the suprabasal layers, normally quiescent in control skin (Figure 1D). Tg epidermis contained an abundance of cells expressing progenitor markers such as K5 and K14, whereas expression of the differentiation markers K10 and loricrin was mostly abrogated (Figure 1E and Figure S1B). Additionally, the number of cells expressing p63, a SC marker and essential regulator of stemness in stratified epithelia (Senoo et al., 2007), was amplified in Tg skin (Figure 1F). Tg skin failed to express hair follicle SC markers such as K15, or Sox9 (data not shown). These results suggested that activation of Yap1 induced expansion of an undifferentiated stem/progenitor cell population in the interfollicular epidermis. We then compared the clonogenic capacity of primary keratinocytes isolated 7 days after Dox administration. Skin of Tg mice had a >5-fold increase in the number of colony-forming cells compared to controls after Yap1 activation in vivo (Figure 1E). Moreover, serial plating assays, typically used to distinguish self-renewing SCs versus shorter-lived progenitors, revealed that Yap1 activation enhanced the SC proliferative capacity by 10-fold in secondary and tertiary platings (Figure 1G and Figure S1C). Taken together, these experiments demonstrate that activation of Yap1 promotes the proliferation of epidermal stem and progenitor cells and that its regulation is critical in maintaining normal skin homeostasis.

Activation of Yap1 Can Lead to Squamous Cell Carcinoma-like Tumors

Tg mice became ill 8 days after Dox induction, likely because of similar dysplastic changes in mucosal tissues such as the tongue (Figure S1D). In order to assess the long-term effects of Yap1 activation, newborn Tg and control skin was grafted onto Nude-mouse recipients followed by Dox administration. Epidermal thickening, hyperkeratosis, and stunted hair growth were evident in Tg-grafted mice shortly after grafting. Beginning at 20 days, Tg grafts developed large tumor-like masses that subsequently ulcerated (Figure 2A). Histological analyses revealed hyperplasia and multiple epidermal invaginations into the dermis as early as 9 days after grafting (Figure 2B). These lesions were epidermal in nature, as judged by staining with a pan-cytokeratin antibody and by their expression of keratin 6,

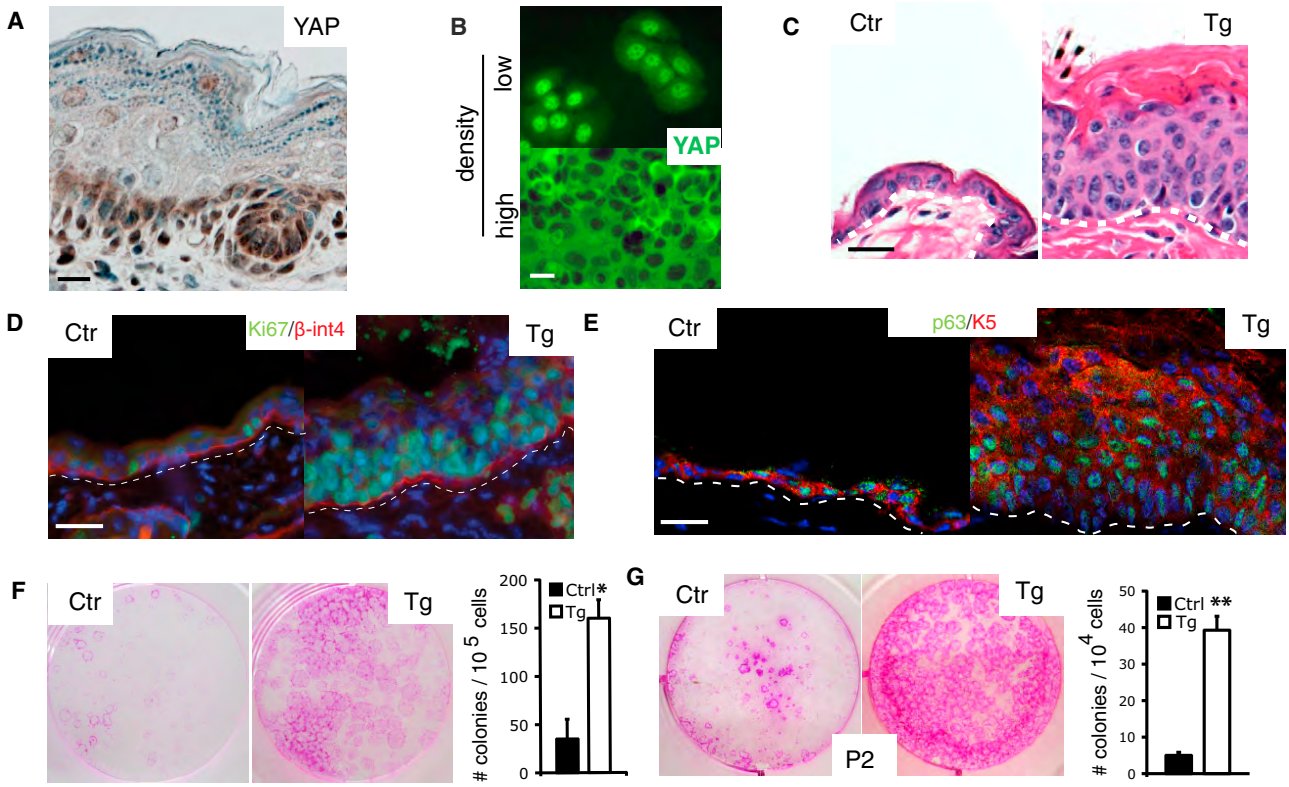

Figure 1. Activation of Yap1 Leads to Epidermal Stem Cell Expansion

(A) Yap1 expression (brown) indicated by immunohistochemical staining on E18.5 murine wild-type epidermis.

(B) Immunofluorescence staining for Yap1 shows a dynamic localization pattern in human keratinocytes that is cell density dependent.

(C) Hematoxylin and eosin (H&E) staining shows an abnormally thick epidermis and hyperkeratinization in adult Tg mouse skin after 8 days of doxycycline (Dox) induction.

(D–E) Immunofluorescence analysis on frozen sections of adult epidermis shows an expansion of the proliferative Ki-67-positive compartment as well as the stem cell compartment (p63-positive, K5-positive) in Tg skin after 8 days of dox induction. Dashed line marks epidermal-dermal junction.

(F) Rhodamine B staining of primary mouse keratinocytes, isolated from 8 day Dox-treated Tg mice and cultured for 9 days on feeders, shows a significant expansion of the epidermal stem cell compartment demonstrated by a higher colony-forming efficiency in Tg skin.

(G) Rhodamine B staining and colony counts show a significant increase in the self-renewal capacity of colony-forming progenitors after Dox administration measured by serial replating assays (shown is passage 2, P2).

Data are presented as mean ±. *$p < 0.05$; **p value < 0.01. Scale bars, 20 μm. See also Figures S1A–S1C.

which is expressed anomalously in hyperproliferative states (Figure 2C). Donor origin of the tumors was confirmed by their expression of the Yap1 transgene (Figure 2D and Figure S1E). Overall, the morphological perturbations resembled those of squamous cell carcinoma (SCC) in situ in human (Figure 2B). By 42 days, full dermal invasion was evident, and this was associated with the rupture of the basement membrane (Figure 2E). At this stage, tumors resembled human sarcomatoid SCC, characterized by the presence of invasive atypical proliferating spindle cells intermingled with keratinized centers (Figure 2B and Figures S1F and S1G). As predicted from their morphology, invading cells stained mostly negative for keratin markers but expressed the mesenchymal marker vimentin, indicating a potential epithelial-mesenchymal transition, a common occurrence in advanced human SCC (Figure 2E). Tumors were also assessed for their renewal by performing secondary transfers by subcutaneous injection into Nude mice. All transplantations gave rise to large masses of similar morphology as their primary counterparts (Figure S1H). Our results here demonstrate that proper regulation of Yap1 activity is essential for tumor suppression in the epidermis.

Loss of Yap1 Results in Failure of Skin Expansion

To test the role of endogenous Yap1 in epidermal biology, we generated a conditional floxed (fl) allele of Yap1 by gene targeting (Figure S2A). Deletion of loxP-flanked exons 1 and 2 of Yap1 after breeding to K14-Cre mice resulted in epidermis lacking Yap1 protein (Figures S2B–S2D). *K14-Cre Yap1$^{(fl/fl)}$* mice will be referred to as cKO mice hereafter. cKO mice were either aborted or died shortly after birth. Gross examination of mutant embryos at E18.5 revealed thinner and fragile skin and absence of epidermal tissue covering the distal part of the limbs (Figure 3A). Assessment of skin permeability using the toluidine blue dye revealed a complete loss of epidermal barrier function in the regions described and around the mouth and nose (Figure 3B). Histological analyses of the proximal limb skin of cKO

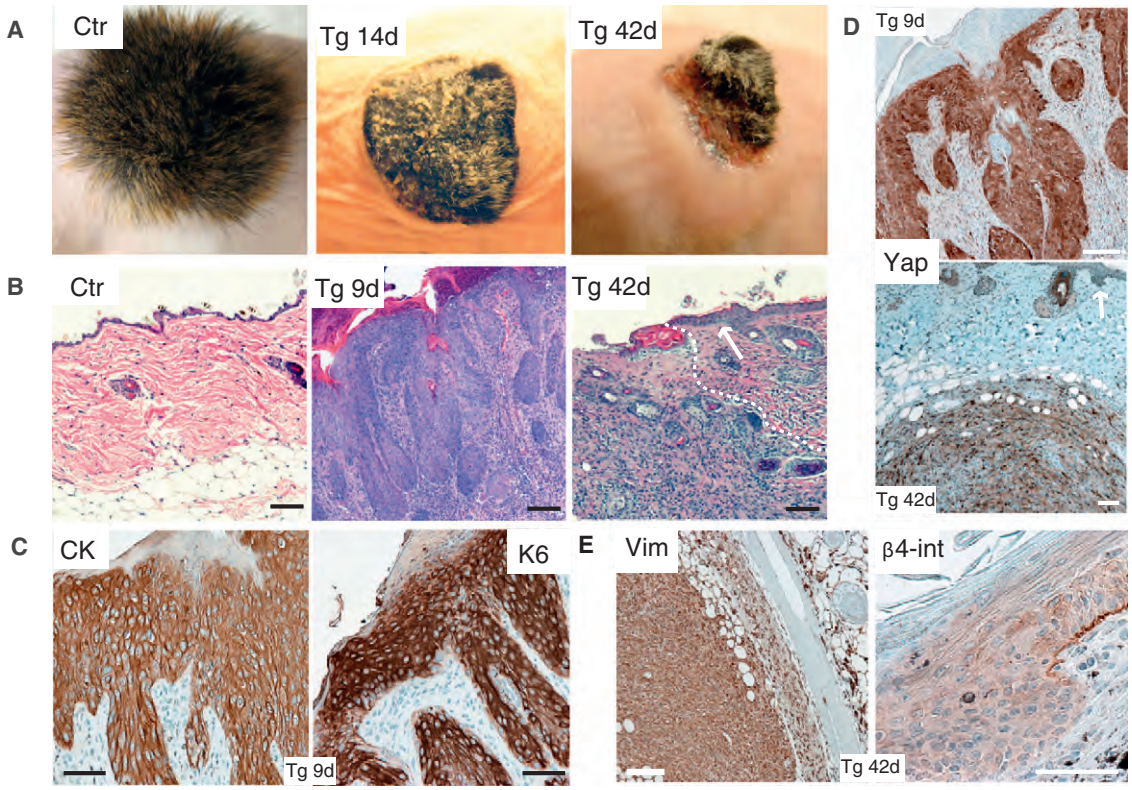

Figure 2. Activation of Yap1 Leads to Tumor Formation

(A) Gross morphology of Ctr and Tg grafts 14 and 42 days after beginning of Dox treatment. Note hyperkeratosis and stunted hair growth in Tg 14d graft and ulceration of skin and subcutaneous tumor mass in Tg 42d graft.

(B) H&E staining of Ctr and Tg grafts 9 and 42 days after Dox induction show an invasion of underlying dermis by invasive keratinocytes derived from the Tg graft. Arrow points out to neighboring normal Nude mouse epidermis. White dashed line indicates border of carcinoma to normal Nude dermis.

(C) Expression of cytokeratin (CK) and keratin 6 (K6) in Tg grafts treated 9 days with Dox as shown by immunohistochemistry.

(D) Immunohistochemistry for Yap1 on Tg grafts with 9 and 42 days Dox treatment reveals epithelial origin of the tumor. Arrow points at Nude mouse epidermis.

(E) Immunohistochemistry for vimentin (Vim) and integrin-β4 (β4-int) on Tg grafts with 42 days of Dox induction.

Scale bars, 100 μm. See also Figure S1D–S1H.

mice revealed a thinner epidermis, reduced stratum corneum, and disorganized epidermal architecture (Figure 3C). Notably, the basal layer was hypoplastic, and basal cells had lost their columnar organization and adopted a flat morphology, typical of suprabasal differentiated cells (Figure 3C). These phenotypes were also observed in the back skin of cKO mice but were less severe.

At E18.5, mutant cells expressing K10, an early differentiation marker, were nearly juxtaposed to the basement membrane in contrast to control skin where the basal layer was more pronounced (Figure 3D). Staining of cKO skin with progenitor markers demonstrated their abnormal morphology and reduction in numbers (Figure 3E). Staining with the proliferation marker phosphohistone H3 (pH3) revealed a >3.5-fold decrease in the number of proliferating basal cells in proximal limb skin of cKO mice (Figure 3F and Figure S2E). No discernible difference in apoptotic cells was detected, as judged by staining with active caspase 3 (data not shown). To directly address whether Yap1 deletion had an effect on the proliferative potential of epidermal SCs, we performed clonogenic assays. These experiments demonstrated a >50-fold decrease in the number of colony-forming cells and a decrease in colony size in cKO epidermis (Figure 3G and Figure S2F). This reduction in proliferative potential is likely the cause of the absence of skin in distal limb areas, where due to growth demands during development, the proliferation rate at E18.5 is more than 3-fold that of back skin (Figure S2E). These results indicate that Yap1 is required for the maintenance of epidermal proliferative potential during development and, together with our gain-of-function experiments, suggest that levels of active Yap1 are a critical and physiological determinant of epidermal SC proliferative capacity.

TEAD Interaction Is Critical for the Role of Yap1 in the Epidermis

We next explored whether the "canonical" Hippo pathway components played a role in regulating Yap1 in the epidermis. In mammals, the transcription factors TEAD1–4 have been shown to interact with Yap1 and to mediate some of its proliferative effects in vitro (Vassilev et al., 2001; Zhao et al., 2008). To test whether TEAD factors were also involved in the regulation of epidermal biology, we generated a reporter construct carrying multimerized copies of the consensus TEAD DNA binding

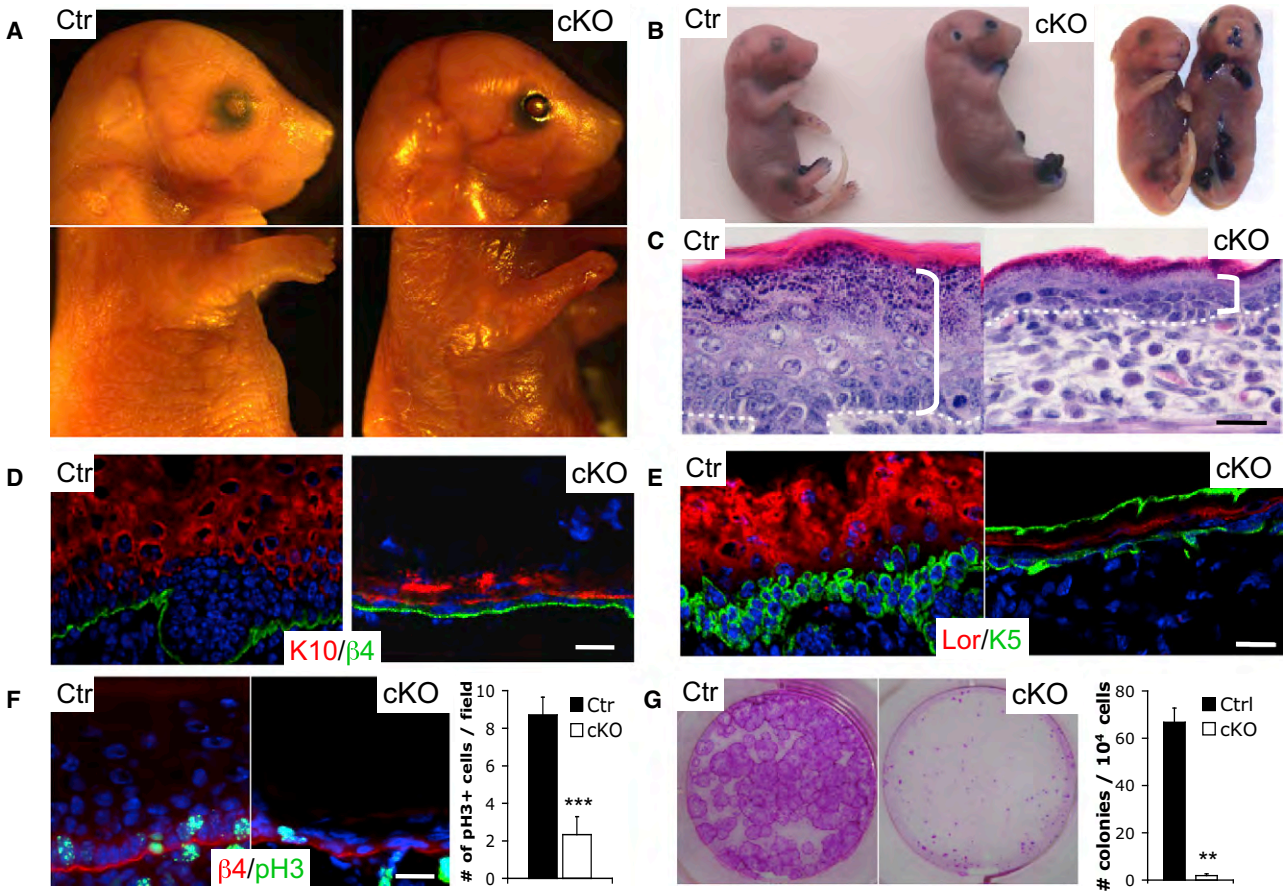

Figure 3. Yap1 Is Required for the Maintenance of the Proliferative Capacity of Epidermal Stem Cells

(A) Gross morphology of representative control (Ctr) and cKO E18.5 embryos. Note absence of skin in distal limbs, eyes, and ears and overall thinner skin.

(B) Toluidine blue skin barrier assay in E18.5 mice reveals absence of epidermal barrier in limbs, ears, nose, and mouth.

(C) H&E staining shows a decrease in epidermal thickness in cKO skin compared to the Ctr (indicated by bracket). Note the flat morphology of basal cells and disorganized epidermal architecture. Dashed line represents epidermal-dermal junction.

(D and E) Immunofluorescence on frozen E18.5 epidermis reveals signs of basal cell depletion in cKO skin. Note the reduction of keratin 10 (K10)-negative, keratin 5 (K5)-positive progenitor/stem cells juxtaposed to the basement membrane (β4). Note the background staining for K5 in stratum corneum of cKO.

(F) Marked reduction in number of proliferative basal cells in cKO limb skin as measured with an antibody against phosphohistone H3 (pH3). Results represent measurements of at least five different fields in three different animals.

(G) Reduction in the number of colony-forming progenitors in E18.5 skin. Rhodamine B stain and colony counts were performed at day 8 after plating and are representative of two independent experiments.

Data are presented as mean ± standard deviation (error bars). $^{**}p < 0.01$; $^{***}p < 0.005$. Scale bars, 20 μm. See also Figure S2A–S2F.

sequence (TBS) to monitor their endogenous activity (Figure 4A). TBS reporter expression in a HaCaT keratinocyte cell line was highly responsive to Yap1, as its overexpression augmented reporter signal and its knockdown (KD) reduced reporter activity (Figure 4A). Proliferating cells in the periphery of colonies expressed the highest levels of the reporter, and reporter signal decreased when these cells were cultured at high densities, thus mimicking the observed pattern of Yap1 activity (Figure 4B).

Given the functional overlap among the four mammalian TEAD proteins, we decided to test their role in epidermal biology in vivo by genetically disrupting their interaction with Yap1. Through homologous recombination in ES cells, we mutated the Serine at position 79 of Yap1 to Alanine (S79A) (Figures S3A and S3B). The ortholog serine in human Yap1 (S94) is necessary to mediate a physical interaction with the TEAD proteins, but not with other DNA-binding cofactors (Zhao et al., 2008). The predicted consequences of this mutation were tested in *Yap1*$^{fl/S79A}$ mouse embryonic fibroblasts after administration of Cre, and we found that, whereas the overall levels of Yap1 protein were unchanged, its ability to interact with TEAD factors was mostly ablated (Figure 4C). We next generated *K14-Cre Yap1*$^{fl/S79A}$ (S79A) mice, in which only the Yap1 mutant protein would be expressed in the epidermis of mice starting at E14.5. These mice died at birth and fully phenocopied cKO mice (Figure 4D). S79A animals lacked epidermis in the distal part of the limbs, eyes, and ears. Mutant limb skin was thinner, with a reduced number of proliferating basal cells lying flat above the basal membrane (Figure 4D and Figure S3C). Clonogenic

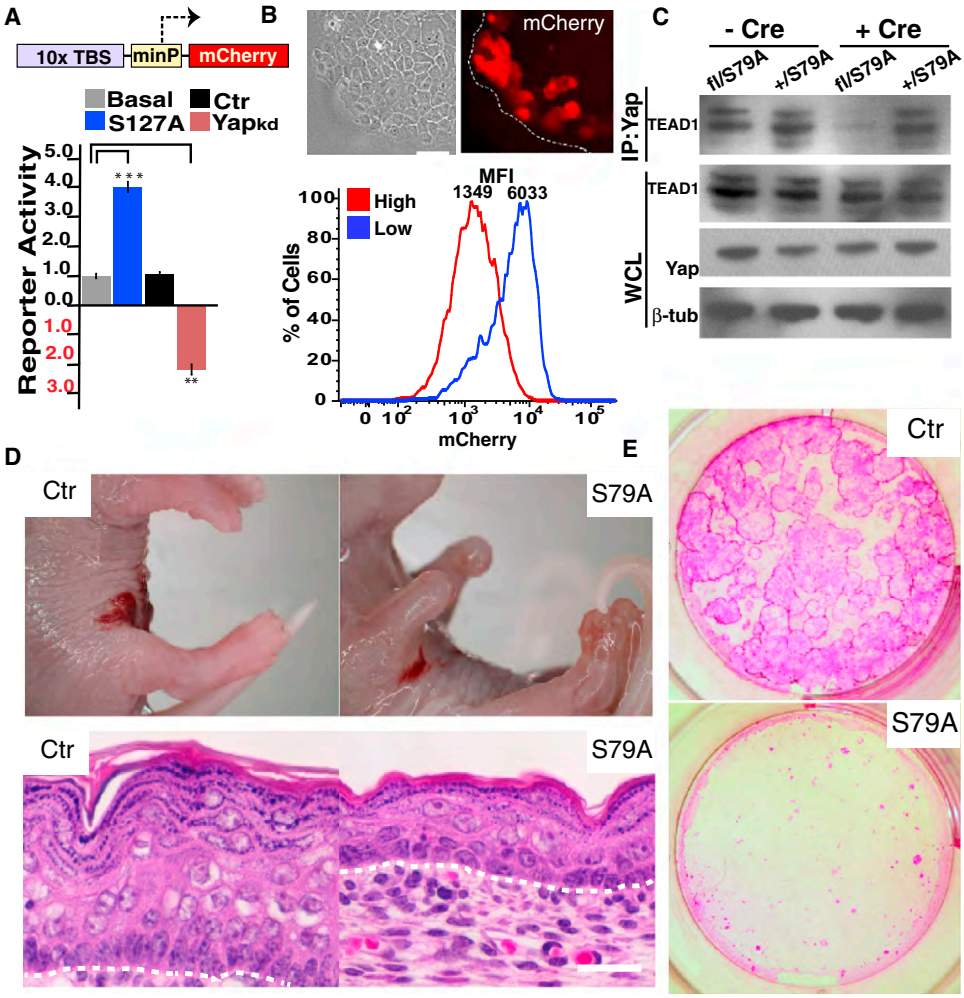

Figure 4. Yap1 Function in the Skin Is Mediated through TEAD Factors

(A) A HaCaT keratinocyte cell line carrying multimerized TEAD-binding sequences (TBS) upstream of a minimal promoter (minP) and an mCherry reporter is responsive to expression of Yap1-S127A, whereas RNAi KD of Yap1 downregulates reporter levels. Cells that have been transfected with a scrambled control-RNAi display reporter activity at basal levels.

(B) TBS reporter dynamics mimic Yap1 activity/localization. (Top) TBS-mCherry shows a dynamic fluorescence pattern with keratinocytes at the edge of a growing colony expressing higher mCherry levels. (Bottom) Reporter signal decreases significantly at high cellular densities.

(C) Physical interaction between Yap1 and TEAD1 is virtually ablated in Adenovirus-Cre (+Cre)-treated $Yap^{fl/S79A}$ embryonic fibroblasts. Immunoprecipitation (IP) was performed with an anti-Yap1 antibody followed by immunoblotting for TEAD1. Loading control β-tubulin (β-tub).

(D) Gross morphology of control (Ctr) and K14-Cre $Yap^{fl/S79A}$ E18.5 embryos. Note the absence of skin in distal limbs and thinner skin with flat basal cells.

(E) Reduction in the number of colony-forming progenitors and proliferative potential in the skin of E18.5 K14-Cre $Yap^{fl/S79A}$ mice, as demonstrated by colony assays stained with Rhodamine B.

Data are presented as mean ± standard deviation (error bars). $^{**}p < 0.01$; $^{***}p < 0.005$. Scale bars, 20 μm. See also Figures S3A–S3C.

assays demonstrated a depletion of SC activity similar to cKO mice (Figure 4E). These results provide genetic evidence that TEADs are likely to be the major transcriptional partners of Yap1 in epidermal proliferation.

α-Catenin Interacts with Yap1 and 14-3-3

The mammalian orthologs of the *Drosophila hippo* kinase, Mst1 and Mst2, function upstream of Yap1 to mediate its phosphorylation and inactivation (Zhao et al., 2007). Deletion of both Mst1 and Mst2 in the liver results in Yap1 activation and hepatomegaly, suggesting that these kinases can act as bona fide negative regulators in vivo (Song et al., 2010; Zhou et al., 2009). We assessed whether deletion of Mst1/Mst2 in the skin would lead to similar phenotypes as Yap1 activation. Surprisingly, the epidermis of mice carrying a skin-specific deletion of Mst1/Mst2 displayed no abnormalities and no evidence of hyperplasia in mice up to 5 months of age (Figures S4A and S4B). This was accompanied by no changes in Yap1 S127 phosphorylation in Mst1/Mst2 null keratinocytes (Figure S4C). Additionally, KD of Mst1/Mst2 in the HaCaT-TBS reporter line produced no change in reporter activity or Yap1 nuclear localization (Figures S4D and S4E). Similarly, knockdowns of Lats1 and

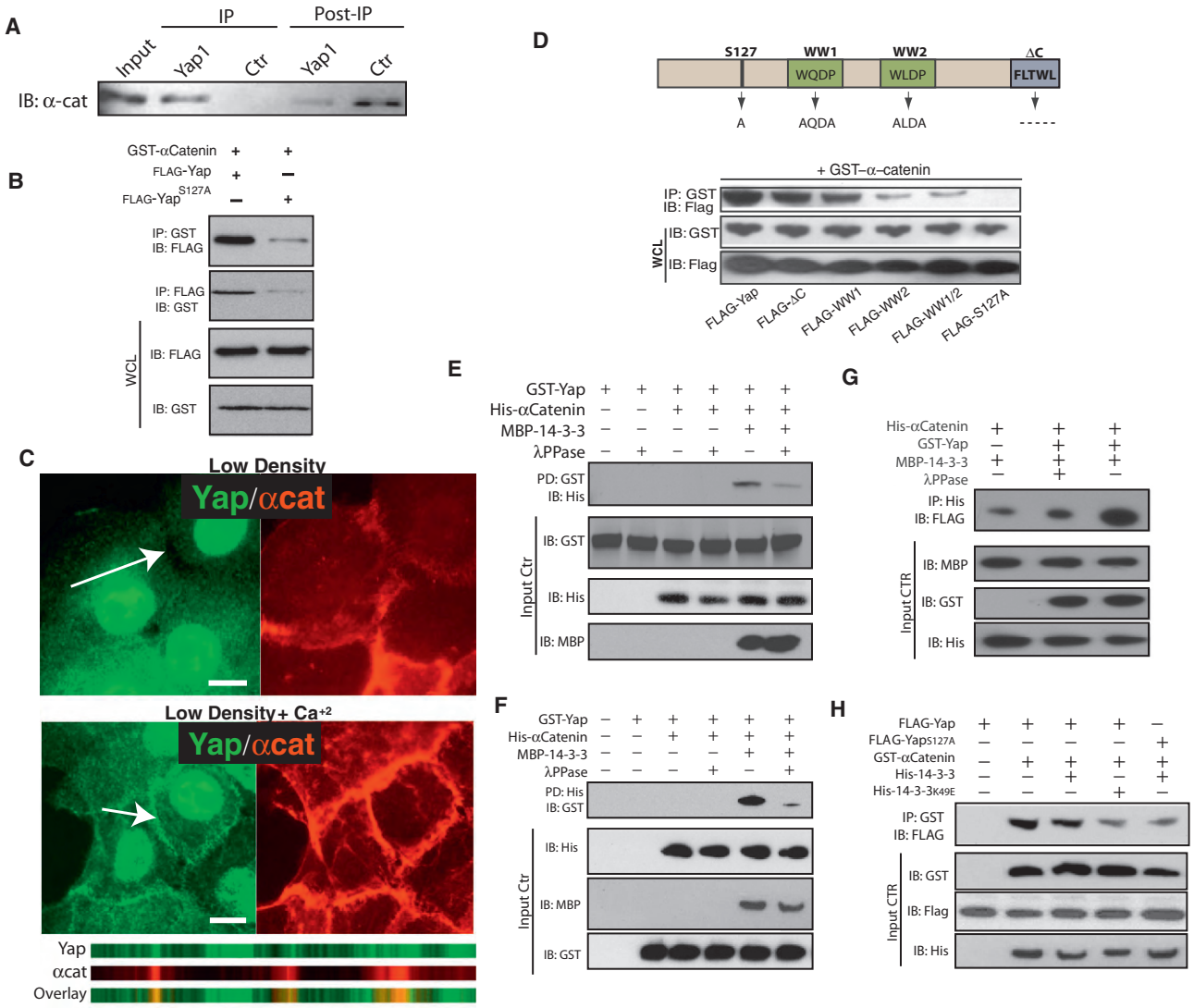

Figure 5. α-Catenin Interacts with Yap1 and 14-3-3

(A) Confluent HaCaT cell lysates (input) were used for an IP with an antibody against Yap1 or Pu.1 (Ctrl) and IP and post-IP material blotted with an antibody for α-catenin.

(B) 293T cells were cotransfected with the indicated plasmids followed by IP with either GST or FLAG-beads. Co-IPs demonstrate an association between full-length Yap1 and α-catenin and a largely diminished association between α-catenin and the YapS127A mutant. IB, immunoblotting; WCL, whole-cell lysate.

(C) Calcium-induced differentiation in primary human keratinocytes leads to translocation of Yap1 into membrane regions that colocalize with α-catenin.

(D) Characterization of Yap1 domains that are important for the interaction with α-catenin.

(E and F) In vitro pull-down (PD) of recombinant Yap1 and α-catenin in the presence or absence of 14-3-3.

(G) In vitro pull-down of recombinant α-catenin and 14-3-3 in the presence of phosphorylated recombinant Yap1.

(H) Immunoprecipitation of Yap1 and α-catenin demonstrates that the biochemical interaction is dependent on 14-3-3.

Scale bars depicted are 10 μm. See also Figures S4A–S4E, Figures S5A and S5B, and Table S1.

Lats2, the orthologs of *Drosophila warts*, did not lead to increased reporter activity or enhanced Yap1 nuclear localization (Figures S4D and S4E). These results suggested that Yap1 might be regulated by alternative mechanisms in keratinocytes that are independent of the predicted canonical Hippo pathway kinases. In order to identify potential alternative upstream regulators of Yap1 activity, we performed coimmunoprecipitation (co-IP) experiments followed by mass spectrometry (MS) analysis in high-density HaCaT cultures. Unexpectedly, this screen revealed that the most common interaction partner of Yap1 in keratinocytes corresponded to α-catenin, an important tumor suppressor in stratified epithelia (Table S1).

We next validated our mass spectrometry data by characterizing the interaction of Yap1 with α-catenin. Endogenous co-IP experiments in confluent HaCaT cells and keratinocytes directly isolated from skin, and pull-downs following transfection of tagged forms of Yap1 and α-catenin in 293T cells further demonstrate the biochemical interaction (Figures 5A and 5B and

Figure S5A). Confocal microscopy studies in primary human keratinocytes corroborate these findings and reveal that Yap1 can begin to colocalize with α-catenin shortly after induction of differentiation following calcium treatment (Figure 5C). With more time in calcium or at high cell densities, nuclear Yap1 is depleted, with some of it colocalizing with α-catenin at the membrane and some accumulating in a cytoplasmic pool (Figure S5B).

Phosphorylation of Yap1 at Ser127 creates a 14-3-3 binding site that has been shown to be crucial for the regulation of its activity (Zhao et al., 2007). Remarkably, disruption of 14-3-3-mediated regulation by mutation of Yap1 Ser127 to alanine nearly abolished the interaction between Yap1 and α-catenin (Figures 5B and 5D). The participation of 14-3-3 in Yap1 binding to α-catenin is further evidenced by the presence of a large number of 14-3-3 peptides found in our Yap1 mass spectrometry pull-downs (Table S1). We also find that the WW domains of Yap1, previously shown to be important for negative regulation by inhibitory kinases (Oka et al., 2008), are also required for its efficient interaction with α-catenin (Figure 5D).

We next examined whether the interaction between α-catenin and Yap1 was direct using in vitro binding assays. Bacterially purified α-catenin and recombinant Yap1 purified from Sf9 cells were coincubated and subjected to pull-down assays. Our results reveal a lack of direct interaction between these two proteins (Figures 5E and 5F). Given that phosphorylation at Yap1 S127 and subsequent 14-3-3 binding are critical for the Yap1 and α-catenin association in cells, we tested whether 14-3-3 could allow for the formation of a tripartite complex in vitro. Indeed, the addition of bacterially purified 14-3-3 resulted in the association of Yap1 and α-catenin (Figures 5E and 5F). As predicted, this association was dependent on Yap1 phosphorylation, as pretreatment of phosphorylated recombinant Yap1 with lambda phosphatase resulted in loss of complex formation (Figures 5E and 5F). Our results also provide evidence for a direct physical interaction between α-catenin and 14-3-3, which can be enhanced by the presence of phospho-Yap1 (Figure 5G). Additionally, expression of a dominant-negative 14-3-3 mutant (K49E) in mammalian cells leads to a reduction of Yap1 and α-catenin binding (Figure 5H). Thus, these data uncover α-catenin as a binding partner for Yap1 and demonstrate that 14-3-3 is critical for this biochemical interaction.

α-Catenin Regulates Yap1 Localization and Activity

To test whether AJs are functionally important for the nonnuclear localization and inactivation of Yap1 at high cellular densities, keratinocyte cultures were treated with the calcium chelator EGTA. After only 1 min of EGTA treatment, the subcellular localization of Yap1 turned predominantly nuclear (Figure 6A and Figure S5C). The rapid kinetics of these observations indicates that AJs play a functional role in inactivating Yap1 and that the same pool of Yap1 can rapidly relocalize to the nucleus. To more specifically dissect the role of individual AJ proteins in regulating Yap1 activity, we depleted their expression in HaCaT and 293T cell lines carrying the TBS reporter. Remarkably, a robust activation of the reporter was observed after α-catenin KD, similar to levels obtained after depletion of the known upstream regulator NF2. A weaker but significant response was obtained after depletion of p120-catenin, and no reporter activation was observed after KD of β-catenin, E-cadherin, P-cadherin, or both E- and P-cadherins (Figure 6B and Figures S5D and S5E). α-catenin depletion activated the TBS reporter in several other cell lines tested, indicating a conserved phenomenon across epithelial cell types (Figure S5F). Silencing of α-catenin in confluent keratinocytes resulted in Yap1 relocalization to the nucleus and the formation of a Yap1/TEAD nuclear complex (Figure 6C-D). α-catenin depletion was also accompanied by a reduction in the inhibitory phosphorylation at Yap1-S127 and minimal changes in the activation status of the Mst and Lats kinases (Figure 6E), suggesting that the regulatory effects of α-catenin might be independent of these kinases.

To extend these observations and to test whether α-catenin could be an important negative regulator of Yap1 in vivo, we examined Yap1 localization in α-catenin mutant tissues. We first analyzed Yap1 localization in epidermis of E18.5 mice with a K14-Cre driven deletion of α-catenin. Skin of control mice displayed the typical heterogeneous subcellular localization pattern in which only a few basal cells display predominant nuclear Yap1 protein (Figure 6F). This pattern was drastically changed in α-catenin mutant epidermis where basal cells were, in their great majority, positive for nuclear Yap1 and showed substantially enhanced nuclear staining (Figure 6F and Figure S5G). Additionally, in some regions, we observed nuclear Yap1 staining extending into the suprabasal cell compartment. Nuclear Yap1 was never seen suprabasally in control tissue (Figure 6F). We next evaluated the localization of Yap1 in a panel of 19 human skin SCCs that expressed varying levels of α-catenin (Figure S5H). As shown in Figure S5H, no or low expression of α-catenin clearly correlated with enhanced nuclear Yap1 localization in these tumors.

Having established that α-catenin is crucial for the inactivation of Yap1 in keratinocytes in vitro and in vivo, we tested whether ectopic expression of α-catenin could result in the recruitment of Yap1 to AJs. Remarkably, enforced expression of α-catenin in low-density cultures led to the relocalization of Yap1 to membrane regions containing AJ components (Figure 6G and Figure S6A). To test whether a different AJ protein could mediate this recruitment of Yap1 to the membrane, we performed similar experiments with E-cadherin. Cells that overexpressed E-cadherin, however, did not show localization of Yap1 to membrane regions (Figure S6B). These observations support the idea that α-catenin plays a direct role in the recruitment of Yap1 to AJs. This result, together with the siRNA depletion data (Figure 6B), speaks to the unique ability of α-catenin, and not E/P-cadherin or β-catenin, to regulate Yap1 localization and activity. These findings also argue that Yap1 hyperactivation is not due simply to loss of AJ-mediated cellular adhesion.

Genetic ablation of α-catenin in murine epidermis leads to keratinocyte hyperproliferation and squamous cell carcinoma (Kobielak and Fuchs, 2006; Vasioukhin et al., 2001), observations that are highly reminiscent of the Yap1 gain-of-function phenotypes described here. Altogether, our data suggest that the growth-suppressive effects of α-catenin in the epidermis might be mediated through inactivation of Yap1. We tested this prediction experimentally and found that reduction of Yap1 levels in a α-catenin KD HaCaT cell line rescued its

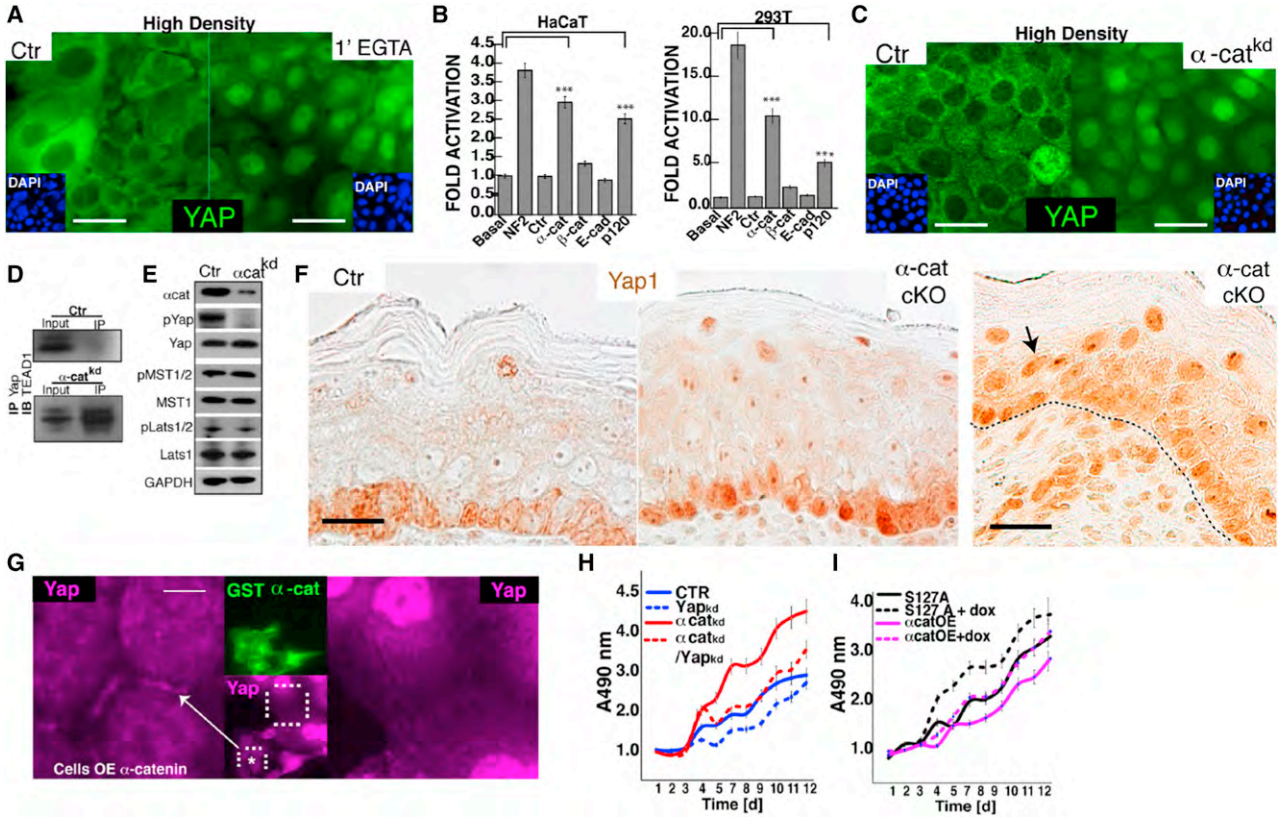

Figure 6. α-Catenin Regulates Yap1 Localization and Activity

(A) Disruption of AJs in high-confluent keratinocytes with EGTA results in Yap1 nuclear localization after 1 min of treatment.

(B) Knockdown (KD) of AJ proteins in either HaCaT or 293T cells carrying the TBS-reporter. KD of NF2 is shown as a positive control.

(C and D) KD of α-cat in confluent keratinocytes leads to Yap1 nuclear localization, and interaction with its nuclear partner Tead1 (D).

(E) siRNA-mediated depletion of α-cat in high-density HaCaT cultures leads to loss of Yap phosphorylation at serine 127 (pYap) and unchanged levels of activated Mst1/2 (pMst) or Lats1/2 (pLats).

(F) Immunohistochemistry for Yap1 in Ctrl and K14-Cre conditional α-catenin mutant (cKO) E18.5 epidermis. Note the enhanced nuclear staining in basal and suprabasal cells of cKO mice.

(G) Immunofluorescence detection of Yap1 (purple) localization in low-density keratinocytes ectopically expressing GST-α-cat (green). Keratinocytes overexpressing α-cat show Yap1 localization to sites of cell-cell contact, whereas untransfected cells show nuclear staining and an absence of Yap1 staining at the cell membrane.

(H) Stable knockdown of α-catenin (α-cat-KD) promotes hyperproliferation in HaCaT cells. However, doxycycline (dox)-induced KD of Yap1 in α-cat-KD cells slows down the rate of cell proliferation to control (Ctr) levels.

(I) Ectopic expression of α-cat in a HaCaT cells suppresses cell proliferation (α-catOE). This growth inhibition is rescued by the expression of a Dox-inducible Yap1S127A mutant (Yap1S127A+Dox).

Data are mean ± standard deviation (error bars). ***$p < 0.001$. Scale bars, 20 μm (A and C), 10 μm (F), and 5 μm (G). See also Figures S5C–S5H and S6A–S6E.

hyperproliferative phenotype to the level of control keratinocytes (Figure 6H and Figures S6C–S6E). Conversely, ectopic expression of α-catenin suppressed keratinocyte proliferation (Figure 6I), but expression of Yap1-S127A was able to rescue these inhibitory effects (Figure 6I).

α-Catenin Binding Prevents Yap1 Dephosphorylation and Activation

Knockdown of α-catenin results in decreased Yap1 S127 phosphorylation, which is likely the cause of increased Yap1 nuclear localization and activity. To begin understanding the potential mechanisms linking α-catenin levels and Yap1 phosphorylation, we performed a Yap1 co-IP followed by MS in α-catenin KD cells. These experiments revealed a selective interaction of Yap1 with the catalytic subunit of the protein phosphatase 2A (PP2Ac) in cells with reduced α-catenin levels (data not shown). Co-IP and immunoblotting experiments confirmed this observation (Figure 7A). These results suggested that loss of α-catenin could result in a more efficient association of Yap1 and PP2Ac, which could potentially dephosphorylate Yap1-S127, triggering its activation. PP2A is a highly promiscuous enzyme that catalyzes the dephosphorylation of a wide range of substrates. Purified PP2Ac, in fact, is able to efficiently dephosphorylate Yap1 S127 in vitro in an okadaic acid-dependent manner (Figure 7B). Importantly, reduction of PP2Ac levels in α-catenin KD cells suppresses the increase in TBS reporter activity and S127A phosphorylation observed after depletion of α-catenin (Figure 7C and Figure S7). Thus, PP2Ac acts downstream or in

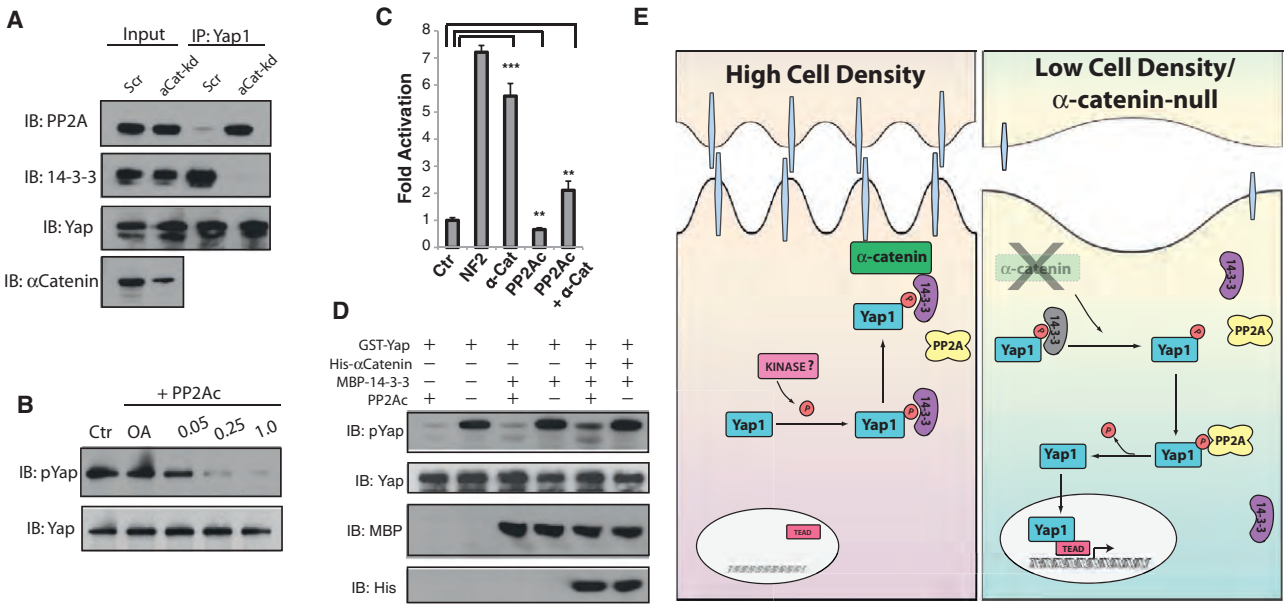

Figure 7. α-Catenin Binding Prevents Yap1 Dephosphorylation and Activation

(A) PP2Ac associates with Yap1 in α-catenin-depleted HaCaT cells. The association of Yap1 and 14-3-3 is diminished in the absence of α-catenin.

(B) Yap1 is dephosphorylated by PP2Ac in vitro. Different concentrations of purified PP2A were incubated with Sf9-purified recombinant Yap1 for 30 min.

(C) siRNA KD of α-catenin increases TBS reporter activation and the dephosphorylation of Yap1. Simultaneous KD of α-catenin and PP2Ac suppresses TBS reporter transcriptional activity and increase in Yap1 phosphorylation.

(D) Western blot analysis of phospho-Yap levels following an in vitro dephosphorylation competition assay of Yap1 by PP2Ac in the presence of recombinant 14-3-3 and α-catenin.

(E) Schematic model of density-dependent and α-catenin-mediated Yap1 activation.

Data are presented as mean ± standard deviation (error bars). $^{**}p < 0.01$; $^{***}p < 0.001$. See also Figure S7.

parallel of α-catenin, and the increased Yap1 activity in α-catenin KD cells is dependent on PP2Ac activity.

An antagonistic and competitive interaction has been reported between PP2A and 14-3-3 for different phosphosubstrates (Chiang et al., 2003; Martin et al., 2008). Hence, we tested whether KD of α-catenin would result not only in increased association of Yap1 with PP2Ac, but also in reduced binding to 14-3-3. In effect, Yap1 does not seem to be bound to 14-3-3 in α-catenin KD keratinocytes (Figure 7A). To determine whether a Yap1, 14-3-3, and α-catenin complex could be protective against PP2Ac-mediated dephosphorylation, an in vitro dephosphorylation assay was performed in the presence and absence of these complex-forming proteins. Though the presence of 14-3-3 alone was not able to mitigate phosphatase activity, addition of α-catenin to the complex resulted in diminished dephosphorylation (Figure 7D).

One of the many PP2A complexes has been previously implicated in the regulation of Hippo signaling by controlling phosphorylation of the Hpo kinase in *Drosophila* (Ribeiro et al., 2010). Our experiments in mammalian cells extend the role of PP2Ac as a regulator of mammalian Hippo signaling and suggest that α-catenin might regulate Yap1 activity by modulating its ability to be dephosphorylated at S127.

DISCUSSION

The present findings provide a mechanistic paradigm for the regulation of SC proliferation and tissue expansion in response to extracellular cues. In this molecular circuitry, cellular "neighborhood" information is provided by density-dependent cell-cell junctions (Figure 7E). A critical and unique component of these junctions, α-catenin, acts as a signaling molecule to translate this context-dependent information into SC proliferation and tissue expansion. α-catenin achieves this through modulating the activity of Yap1, the transcriptional coactivator of the size-restricting Hippo signaling pathway.

Yap1 as a Critical Regulator of Epidermal SCs

Relatively little is known about the transcriptional networks controlling epidermal SC maintenance. Arguably, only p63 and Tcf3/Tcf4 have been implicated as essential factors for the long-term proliferative capacity of epidermal SCs in vivo (Nguyen et al., 2009); Senoo et al., 2007). Our work here provides loss- and gain-of-function genetic evidence indicating that Yap1 and the TEAD proteins are critical endogenous regulators of epidermal SC and progenitor proliferation. While this manuscript was under review, Zhang et al. reported that transgenic activation of Yap1 in the skin led to an expansion of basal epidermal progenitors and inhibition of terminal differentiation (Zhang et al., 2011). These authors also find that nuclear Yap1 progressively declines with age and that it correlates with the proliferative potential of epidermal progenitors. Our work corroborates and extends these observations, and together they highlight the emerging role of Yap1 and TEAD factors in skin homeostasis. In the future, it will be important to identify

the transcriptional targets of this complex that mediate its function.

Our results also extend previous observations linking Yap1/TEAD activity to SC function. In the intestine, Yap1 activation leads to the expansion of an undifferentiated progenitor population (Camargo et al., 2007), whereas its deletion causes no obvious defects during development or homeostasis, though it impairs regeneration (Cai et al., 2010). Loss of Yap1 in the liver leads to defective biliary development but unimpaired regeneration after partial hepatectomy (Zhang et al., 2010; F.D.C., unpublished data). Thus, it seems that the requirement for endogenous Yap1 in tissue-specific SCs varies significantly according to the cellular context. The intrinsic need for Yap1/TEAD activity in epidermal SCs during development is more akin to the role that this complex plays in human embryonic SCs, in which it is required for their self-renewal and suppression of differentiation (Lian et al., 2010).

α-Catenin as a Regulator of Yap1 Activity

Our findings provide additional insight into the role of α-catenin in growth and tumor suppression. Previously, α-catenin was considered to be a simple structural linker between the cadherin/catenin complex and the actin cytoskeleton. During the past several years, it has become evident that α-catenin also functions as a signaling molecule in a variety of cellular processes in addition to its role in cellular adhesion (Stepniak et al., 2009). Interestingly, ablation of epidermal E- and P-cadherins leads to AJ disruption, defects in intracellular adhesion, and epidermal integrity but no significant increase in cellular proliferation (Tinkle et al., 2008). These findings, together with our data (Figure 6B and Figure S5D), demonstrate that hyperproliferation and Yap1 activation in the absence of α-catenin are not simply due to loss of cell adhesion and epithelial integrity. Additionally, in human cancers, loss of α-catenin is usually a stronger prognostic factor than loss of E-cadherin (Benjamin and Nelson, 2008). Thus, though it seems that AJ components function coordinately in mediating keratinocyte adhesion, they differ in their ability to influence proliferative responses. Particularly, it was shown that aberrant activation of the Ras-MAPK pathway was partially responsible for the hyperproliferative phenotypes observed in α-catenin mutant skin (Vasioukhin et al., 2001). It is still unclear to what extent Yap1, Ras-MAPK, or other signaling pathways contribute to growth suppression mediated by α-catenin in the skin. Whether Yap1 mediates all or only part of α-catenin tumor-suppressive activity awaits the results of epistatic experiments in vivo.

14-3-3 and Yap1 Phosphorylation in the Skin

Our data would suggest that 14-3-3 proteins are also critical growth suppressors in the epidermis. The 14-3-3 proteins are a large group of highly conserved and homologous dimeric proteins. A particular isoform, 14-3-3σ, is unique among the family in that it is selectively expressed in stratified epithelia. Interestingly, 14-3-3σ is barely detectable in basal cells but is abundantly expressed in the suprabasal layer of human epidermis (Dellambra et al., 2000). Downregulation of 14-3-3σ in primary keratinocytes results in the expansion and immortalization of cells expressing p63 (Dellambra et al., 2000; Pellegrini et al., 2001). Additionally, the epidermis of mice carrying a dominant-negative mutation in 14-3-3σ displays hyperplasia, expansion of K14 and K5-expressing progenitor cells, and enhanced proliferative capacity in vitro (Herron et al., 2005; Li et al., 2005). These phenotypes are reminiscent of Yap1 overexpression and α-catenin deletion in the epidermis and further indicate that 14-3-3σ is part of a critical molecular circuitry controlling epidermal progenitor expansion.

As predicted from work in *Drosophila*, Mst1/2 and Lats1/2 are critical for Yap1 S127 phosphorylation in a wide array of cell lines (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007). Our data here demonstrate that Mst1/2, and potentially Lats1/2, are dispensable for Yap1 phosphorylation and activity in the epidermis. Our results parallel those of Zhao et al. and identify an unexpected diversity in the mammalian kinase components that are necessary to phosphorylate Yap1 in relation to what is predicted from work in *Drosophila*. For instance, in mouse liver, Mst1/2 signal through an unknown intermediary kinase that is distinct from Lats1/2 to control Yap1 phosphorylation. On the other hand, mouse embryonic fibroblasts do not require Mst1/2 but do depend on Lats1/2 to phosphorylate Yap1 (Zhao et al., 2007). We have tested whether pharmacological manipulation of MAP kinase and insulin signaling, pathways that were previously shown to be involved in α-catenin signaling (Vasioukhin et al., 2001), had effects on Yap1 phosphorylation. Inhibition of IGFR, JNK, and Src kinases resulted in no relative change in Yap1-S127 phosphorylation (data not shown). Thus, the identity of the kinases that are responsible for Yap1 inactivation in the epidermis remains unknown.

Several examples exist in the literature that demonstrate that competition between 14-3-3 proteins and PP2A complexes for a substrate is critical for regulation (Chiang et al., 2003; Martin et al., 2008). Our data suggest that binding of the Yap1/14-3-3 complex to α-catenin would stabilize this complex and/or inhibit access of PP2Ac (Figure 7). When binding to α-catenin is lost, PP2Ac could then more efficiently compete for binding to S127 in Yap1 and could dephosphorylate it, allowing Yap1 to translocate into the nucleus, bind to TEADs, and activate a proproliferative gene expression program (Figure 7). Recently, α-catenin has been found to interact with NF2, another upstream component of the Hippo pathway (Gladden et al., 2010). Interestingly, it was reported that NF2 could link α-catenin to Par3, thus providing a bridge between AJs and polarity complexes. Together with the recent identification of the apical polarity protein Crumbs as a functional regulator of the Hippo pathway (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010; Varelas et al., 2010), these findings raise the intriguing possibility that a signaling complex of α-catenin, NF2, and other cell polarity proteins that are associated with the cell membrane could make up an important signaling hub that may allow cells to sense cues such as density and positioning. These complexes then could relay those signals to transcriptional effectors such as Yap1 (see below). The LIM protein Ajuba might provide an additional avenue for crosstalk between AJs and Hippo signaling. Ajuba can interact with α-catenin and has been recently shown to inhibit Yap1 phosphorylation via interaction with Lats (Das Thakur et al., 2010). Further studies are needed to determine whether and to what extent α-catenin, NF2, and polarity complexes in epidermal cells control proliferation through Yap1.

Crowd Control and the Hippo Pathway

An important longstanding question in the Hippo signaling field is the identity of extracellular signals that modulate the pathway's activity. It has been speculated that this pathway could be involved in interpreting morphogen gradients to regulate tissue size, and therefore the existence of a secreted or membrane ligand has been postulated (Harvey and Tapon, 2007). Our data here suggest that, in the epidermis, this regulation is, at least partly, mediated by α-catenin and AJs. Based on the massive overgrowth phenotypes obtained by deletion of α-catenin in the skin and the developing brain, Vasioukhin et al. postulated that AJs could act as molecular biosensors of cell density and positioning (Lien et al., 2006a, 2006b). Our data here support and extend this notion and indicate that Yap1, through its interaction with α-catenin, is a critical mediator of this "crowd control" molecular circuitry in the epidermis (Figure 7). In this model, increased cellular density or differentiation (which is sensed by an increased number of AJs), higher calcium concentration (Menon and Elias, 1991), and selective expression of 14-3-3 in the suprabasal layers limit SC expansion by inactivating Yap1. Low basal cell density, as in a growing embryo or after wounding, would translate into nuclear Yap1 and proliferation. When this molecular network is defective, e.g., by deletion of α-catenin, inactivation of 14-3-3, or activation of Yap1, hyperproliferation and tumors can arise. Further elucidation of the transcriptional program controlled by Yap1 and identification of modulators of its interaction with α-catenin, including the kinase that mediates Yap1 inactivation in keratinocytes, will likely provide insights into the mechanisms that underlie SC maintenance, tissue expansion, and tumorigenesis.

EXPERIMENTAL PROCEDURES

Generation of Gene-Targeted Mice

For generation of cKO mice, a targeting strategy was designed to allow Cre-mediated deletion of exon 1 and 2 of *Yap1*. Progeny carrying the targeted Yap1 allele were bred to *K14-Cre* mice. To generate the mutated $Yap1^{S79A}$ allele, a point mutation was introduced by site-directed PCR mutagenesis in the *mYap1*-targeting construct substituting a serine at amino acid position 79 (TCC) for an alanine (GCG). Mice were generated in the same way as described for the $Yap1^{fl/fl}$ mice. Mice carrying the $Yap1^{S79A}$ allele were bred to *K14-Cre* $Yap1^{fl/+}$ mice to generate *K14 Cre* $Yap1^{S79A/fl}$ mice.

Cell Isolation, In Vitro Culture, and Clonogenic Assays

Primary keratinocytes isolated from the epidermis of E18.5 control and transgenic mice were cultured on mitomycin-C-treated 3T3 feeder cells in FAD medium. For clonogenic assays, colonies of primary mouse keratinocytes were fixed and stained with 1% rhodamine B (Sigma) 9 days after initial plating.

Skin Engraftments and Barrier Function Assay

Full thickness skins of E18.5 K14-Cre $Yap1^{fl/fl}$ and littermate Ctr mice were removed from torsos and spread on a sterile petri dish and briefly stored at 4°C. During this time, each skin graft recipient site was prepared by removal of a patch of full thickness skin on the back of an anesthetized $Foxn1^{nu}$/$Foxn1^{nu}$ mouse. For skin barrier assays, newborn mice were rinsed in PBS and successively dehydrated in methanol for 1 min each. Mice were then rehydrated with PBS and stained in 0.1% toluidine blue in PBS, destained with PBS for 20 min, and then photographed.

Mass Spectrometry and Proteomic Analysis

Approximately 4 mg of precleared high-density HaCaT cells were used for Yap1 immunoprecipitation. Immunoprecipitates were loaded onto SDS-PAGE and stained with colloidal Coomassie. Mass spectrometry analysis was performed at the proteomics facility at Children's Hospital Boston.

Dephosphorylation Assay

Catalytically active recombinant PP2A (PP2Ac) was purchased from Millipore. Recombinant proteins were incubated in the presence of varying concentrations of PP2Ac. For PP2Ac and 14-3-3 competition assays, Yap1, α-catenin, and 14-3-3 were preincubated in 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, and 0.02% Triton X-100 for 1 hr prior to PP2Ac addition.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.cell.2011.02.031.

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EXTENDED EXPERIMENTAL PROCEDURES

Generation of Gene-Targeted Mice

For the generation of cKO mice, a targeting strategy was designed to allow Cre-mediated deletion of exon 1 and 2 of *Yap1*. A targeting construct carrying a neomycin selection cassette and a diphtheria toxin gene was generated by long-range PCR amplification of BAC DNA containing the genomic regions encompassing Yap1. The AatII linearized targeting construct (Figure S1) was electroporated into V6.5 mouse embryonic stem cells. After eight days of G418 selection, 113 individual colonies were screened for homologous recombination by PCR. The 3′ junction of targeting vector and endogenous Yap1 was confirmed using P3 (5′-GATCGAGATCCG AAGTTCCT-3′) and P4 (5′ ACCACCTGTAATGAGATCTGAT-3′), generating a 1.5kb band in case of correct integration. To control for random integration the absence of the vector backbone at the 5′ junction was confirmed with P5 (5′-CGAACGACCTACACC GAACTG-3′) and P6 (5′-AGGAGGATTTCAGCAAGTGCA-3′). One embryonic stem cell clone containing the floxed *mYap1* allele was injected in blastocysts of C57BL/6 mice to generate chimeric mice carrying the *Yap1* targeted allele. Chimeric mice were bred to germline transmission and then mated to Act-Flp mice to remove the neo-selection cassette. Progeny carrying the targeted Yap1 allele were bred to *K14-Cre* mice. To generate the mutated $Yap1^{S79A}$ allele a point mutation was introduced by site-directed PCR mutagenesis in the *mYap1* targeting construct with deleted loxP sites, substituting a serine at amino acid position 79 (TCC) for an alanine (GCG), and verified by DNA sequencing analysis. Mice were generated in the same way as described for the $Yap1^{fl/fl}$ mice. Mice carrying the $Yap1^{S79A}$ allele were bred to *K14-Cre* $Yap1^{fl/+}$ mice to generate *K14 Cre* $Yap1^{S79A/fl}$ mice.

Cell Isolation, In Vitro Culture, and Clonogenic Assays

Primary keratinocytes isolated from the epidermis of E18.5 control and transgenic mice were cultured on mitomycin-C-treated 3T3 feeder cells in FAD medium (DMEM: F12, 3:1) with 100 U/ml penicillin, 50 μg/ml streptomycin, and supplemented with 10% chelexed fetal bovine serum, 5 μg/ml insulin, 10 ng/ml recombinant human epidermal growth factor, 10^{-10} M cholera toxin, 1.8 x10^{-4} M adenine, and 0.4 μg/ml hydrocortisone. For clonogenic assays colonies of primary mouse keratinocytes isolated as described above were counted, fixed and stained with 1% rhodamine B (Sigma) nine days after initial plating. Normal human epidermal keratinocytes (Lonza) were cultured in KBM-2 media with KGM-2 bullet-kit singlequot supplements.

Generation of Cell Lines

Immortalized human keratinocytes (HaCaT) and HEK293T cells were cultured in DMEM+10%FBS. HaCaT and HEK293T cells were both transfected with the TBS-mCherry constructs and selected on puromycin. Cell clones were isolated and tested for mCherry expression and reporter responsiveness to positive and negative controls. α-catenin knockdown HaCaT cells (A7) were created by shRNA infection of α-catenin GIPz (Open Biosystems). Wild-type HaCaT cells and A7 cells were infected with three different clones of an inducible Yap-TRIPz shRNA (A, B, C, Open Biosystems) that express RFP upon Dox-induction (1 mg/ml). Cells were placed on doxycycline for 3 days post-infection and then sorted by flow-cytometry. Stable cell lines are hereto referred to by: HaCaT-A, HaCaT-B, HaCaT-C, A7A, A7B, and A7C. Similarly, HaCaT cells expressing an inducible YapS127A (S127A) were transfected with pDEST27-α-catenin and selected on neomycin, referred to as αcatOE. Mouse embryonic fibroblasts were isolated from $Yap1^{S79A/fl}$ and $Yap1^{S79A/+}$ mice and administered Adenovirus-Cre recombinase for 4 days prior to analysis.

Cell Proliferation Assays

HaCaT cell-lines stably expressing a Dox-inducible Yap shRNA/α-catenin shRNA and doxycycline inducible YapS127A/α-catOE, as described above, were monitored for cell proliferation upon induction with doxycycline (1 mg/ml) with the MTS CellTiter 96 AQ assay kit (Promega) according to the manufacturer's directions.

Subcutaneous Injection of Tumor Cells

For the subcutaneous injection of tumor cells tumors were isolated, minced, digested rotating for 1h at 37°C with 2mg/ml Collagenase/Dispase (Roche) and then filtered twice (100 μm, then 40 μm). 10^4 and 10^5 cells/100 μl matrigel were then injected subcutaneously.

Immunofluorescence, Immunohistochemistry, Immunoblotting, and Antibodies

For immunofluorescence tissues were frozen, embedded in OCT compound, and sectioned (10 μm). Frozen sectioned tissue was fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at RT. Slides were blocked with PBS, 0.2% Triton X-100, 2% BSA, and 5% normal goat serum for 1h at RT. Primary antibody was diluted in PBS, 0.1% Triton X-100, 1% BSA and 2.5% normal goat serum. The following antibodies were used: Yap1 (1:25; Cell Signaling), Ki-67 (1:1000, Leica NCL-Ki67p), phospho-Histone H3 (1:200; Millipore, 04-1093), p63 (1:100; Santa Cruz sc-8343), Keratin 10 (1:500; Covance PRB-159P), Keratin 14 (1:1000; Covance PRB-155P), Keratin 5 (1:1000; Covance PRB-160P), Keratin 15 (1:1000; Covance PCK-153P), Loricrin (1:500; Covance PRB-145P), CD104/Integrin-4β (1:100, BD 553745). For immunohistochemistry, tissues were fixed in 4% paraformaldehyde and then dehydrated and embedded in paraffin. Sectiones were stained for Yap1 (1:50; Cell Signaling). Immunocytochemistry was performed with the following primary antibodies/dilutions/sources: Yap1 (1:1000, Cell Signaling #4912 or Santa Cruz sc101199), α-catenin (1:250, Cell Signaling #3236 or Santa Cruz sc1495), p120-catenin (1:250, BD 610133), GST (1:1000, Cell Signaling #2624). Western blot

analysis was performed with the following primary antibodies/dilutions/sources: TEAD1 (1:500, BD 610923), Yap1 (1:1000, Santa Cruz sc101199), pYap (1:1000, Cell Signaling #4911), α-catenin (1:1000, Cell Signaling #3236), pMST1/2 and MST1 (1:500, Cell Signaling #3681S and #3682), MST2 (1:1000, Avruch lab), GST (1:1000, Cell Signaling #2624), FLAG (1:1000, Sigma 3165), β -tubulin (1:5000, Cell Signaling #5436), GAPDH (1:10,000, Cell Signaling #3683) and CDK4 (rabbit, 1:500; Santa Cruz). Co-immunoprecipitation experiments were performed by lysing cells in RIPA buffer followed by pre-clearing with protein A/G agarose beads. Primary antibodies of interest were added, and Protein:Antibody complexes were incubated overnight at 4°C, and captured on protein A/G beads. Samples were then eluted in laemmli buffer and analyzed by Western blot.

Transfection of siRNA Oligos and Plasmids

HaCaT and 293T cell lines were transfected with 5 nM siRNA (*silencer select*, Ambion) using RNAiMAX (Invitrogen) according to the manufacturers directions. Reporter cell lines were analyzed approximately 4 days post-transfection. Transfection of plasmids was performed using either FUGENE6 (Roche) or Lipofectamine 2000 (Invitrogen) according to manufacturers directions and assayed 48-72 hr following transfection.

Mass Spectrometry and Proteomic Analysis

HaCaT cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate, 1% Triton X-100, protease inhibitor cocktail-Roche), and pre-cleared in protein A/G agarose. Lysates were incubated overnight with anti-Yap1 (Cell Signaling) -chemically coupled beads. Beads were washed in 50 mM Tris, 200 mM NaCl, and protein complexes were eluted in 100 mM Glycine, pH 2.0 and neutralized in 2M Tris, pH 8.5. Centricon Plus-70, Ultracel-PL Membrane, 10 kDa (Millipore) was utilized to concentrate the eluted samples.

Reporter Gene Assay

Reporter assays were carried out in HaCaT and HEK293T cells stably expressing multimerized copies of the consensus DNA-binding sequence for TEAD (TBS) upstream of a minimal promoter driving the expression of mCherry. Analysis of mean fluorescence intensity was carried out by flow cytometry.

In Vitro Binding Assay

Baculovirus expressed recombinant GST-Yap1 was purchased from Abnova. MBP-14-3-3 and His-α-catenin plasmids were purchased from Geneocopia. MBP-14-3-3 and His- α -catenin were expressed and purified with 0.1mM IPTG for 6 hr followed by bacterial lysis and incubation with nickel or amylose resin beads. In vitro pull down assays were performed in the presence of protease inhibitors in binding buffer (50mM Tris (pH 7.4), 150 mM NaCl, 0.02% Triton X-100). Protein complexes were incubated overnight at 4°C followed by 1 hr incubation with subsequent beads.

Adherens Junction Disruption by Calcium Chelation

HaCaT cells grown on glass coverslips to 100% confluency were incubated at 37°C with 4 mM calcium chelation (EGTA) in calcium-free FAD medium over indicated periods of time. Samples were immediately fixed in 4% paraformaldhyde and subjected to immunofluorescence analysis.

Statistical Analysis

For all quantified data, mean value ± one standard deviation was presented. The statistical significance between two experimental groups is indicated in the figures by asterisks, and comparisons were made using the Student's t test. Calculated P-values less than or equal to 0.05 were considered to be of statistical significance.

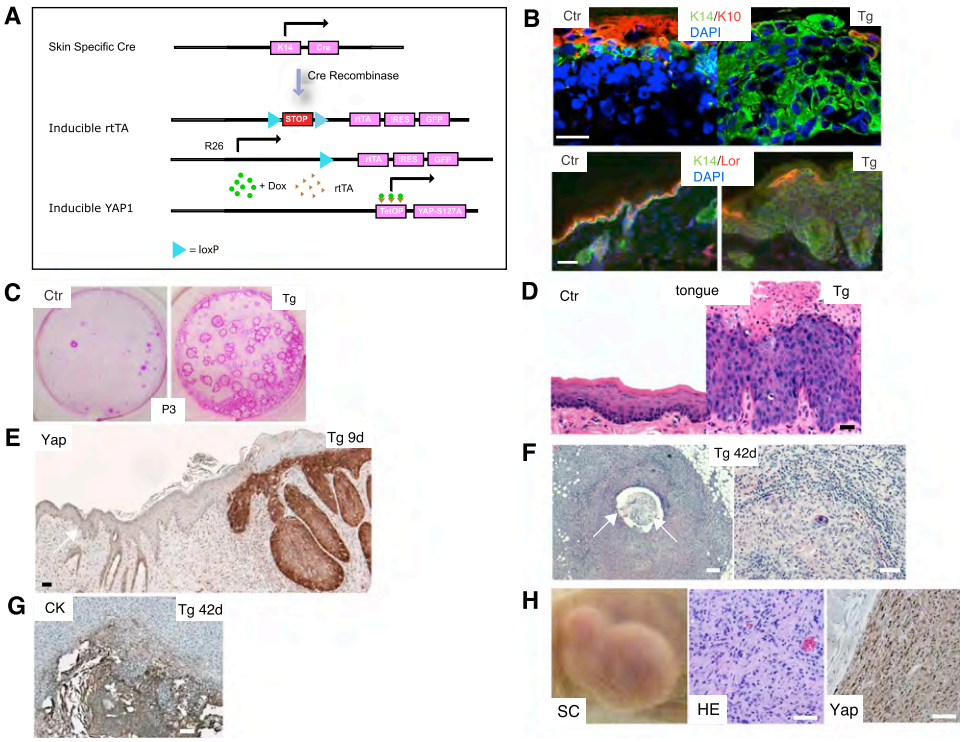

Figure S1. Hyperproliferation and Carcinoma in Skin of Tg Mice, Related to Figure 1 and Figure 2

(A) Genetic strategy for temporal spatial regulation of Yap1-S127A in epidermal cells. K14-Cre deletion of transcriptional STOP cassette in R26 locus allows doxycycline-(Dox) inducible expression of Yap1-S127A in K14-expressing cells and their progeny.

(B) Immunofluorescence analysis shows an expansion of epidermal progenitor cells in adult Tg and control mice after eight days of Dox administration. Note expansion of basal cells (K14), and reduction in differentiated cells (K10 and loricrin/Lor).

(C) Rhodamine B staining reveals an increase in self-renewal capacity of keratinocyte progenitors as shown by an increased number of colonies in the third passage (P3) of Tg keratinocytes compared with the Ctr.

(D) H&E staining of Ctr and Tg tongue eight days after Dox induction reveals thickening and dysplasia of Tg epithelium.

(E) Immunohistochemistry for Yap1 on a Tg graft 9 days after Dox induction showing the transition from nude mouse epidermis (arrow) to Tg skin.

(F and G) Tg 42d tumors are comprised of invading fibroblast-like keratinocyte tissue with sites of keratin deposition (arrows) and stain positive for cyto-keratin (CK).

(H) Subcutaneous (SC) Injection of tumor cells into nude mice followed by Dox treatment for 8 weeks. From left to right: gross morphology of the subcutaneous tumor; H&E staining; immunohistochemistry for Yap1 expression. Scale bars represent 20 μm.

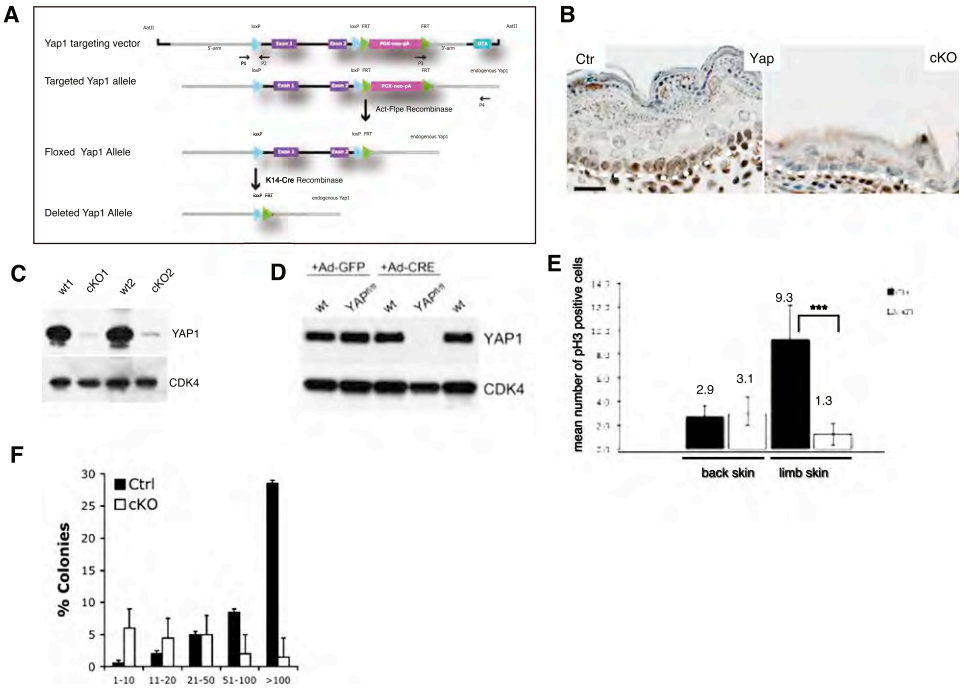

Figure S2. Reduction in Proliferative Capacity of Epidermal Progenitors in the Absence of Yap1, Related to Figure 3

(A) Gene targeting strategy for conditional deletion of exon 1 and exon 2 of murine *Yap1*.

(B) Immunohistochemistry for Yap1 in limb skin sections of Ctr or K14-Cre Yap1$^{fl/fl}$ E18.5 embryos. Scale bar represents 20 μm.

(C) Immunoblot analysis for Yap1 in cultured primary mouse keratinocytes from control (wt) or K14-Cre Yap1$^{fl/fl}$ E18.5 embryos (cKO).

(D) Immunoblot analysis for Yap1 in mouse embryonic fibroblasts isolated from wild-type control or littermate Yap1$^{fl/fl}$ embryos. Cells were transduced with adenoviruses expressing GFP or CRE four days before cell lysis.

(E) Immunofluorescence analysis and counts of pH3-positive cells in the basal layer shows a reduction in number of proliferative basal cells in cKO limb skin but not in back skin of Yap1$^{fl/fl}$ K14-Cre mice. Note that the proliferative index in limb skin is three times as high as that in back skin. Data are presented as mean ± standard deviation (error bars). ***p value < 0.001 calculated by two-tailed Student t test.

(F) Reduction in the number of colony-forming cells in K14-Cre Yap1$^{fl/fl}$ mice. The number of cells in each colony was counted at day nine after initial plating. Data are presented as mean ± standard deviation (error bars).

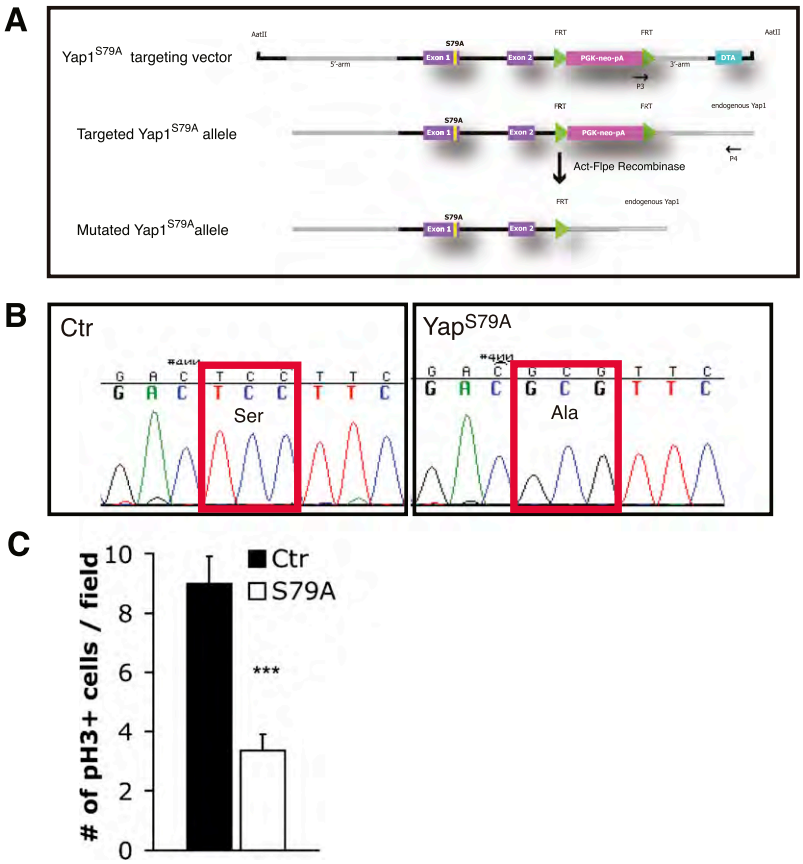

Figure S3. Generation of Yap1-S79A-Targeted Allele, Related to Figure 4

(A) Gene targeting strategy for the introduction of two base pair changes in exon 1 of *Yap1* that leads to an amino acid change at residue 79 from Serine to Alanine.

(B) Sequencing analysis of exon1 cDNA amplified from primary control (Ctr, Serine 79) or K14-Cre $Yap1^{S79A/fl}$ (Yap^{S79A}, Alanine 79) cultured keratinocytes confirms the presence of the mutation at the mRNA level in mutant cells.

(C) Marked reduction in number of proliferative basal cells in in K14-Cre $Yap1^{S79A/fl}$ limb skin as measured by IF with an antibody against phospho-histone H3 (pH3). Results represent the mean of three experimental replicates (5 fields of view) ± standard deviation. ***$p < 0.001$.

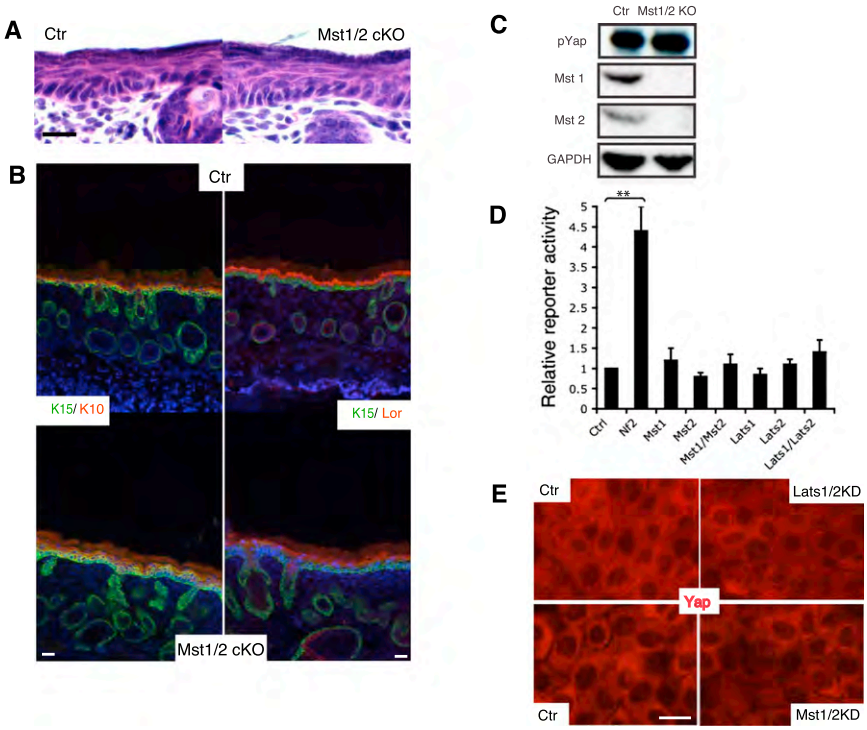

Figure S4. Ablation of Mst1 and Mst2 Does Not Affect Epidermal Development, and Mst and Lats Kinases Do Not Regulate TEAD-Based Reporters, Related to Figure 4

(A) H&E stains of tissue sections from the back skin of postnatal day four (P4) control and K14-Cre $Mst1^{-/-}$ $Mst2^{fl/fl}$ mutant mice indicate no apparent difference.

(B) Immunofluorescence staining for basal (K15) and differentiation (K10, Lor) markers reveals normal differentiation in the skin of P4 mutant mice.

(C) Immunoblot analysis for pYap, Mst1 and Mst2 in primary Mst1/2 cKO keratinocytes.

(D) A HaCaT cell line carrying a multimerized Tead binding site (TBS)-based reporter construct was transfected with a pool of 3 small interfering RNAs (siRNAs) against the selected the gene(s) described in the bottom of the graph. Ctrl represents a scrambled siRNA, and siRNAs against NF2 are used as a positive control for reporter activation. Note that only the NF2 oligo resulted in significant upregulation of the reporter. Results represent mean of three experimental replicates ± standard deviation. **$p < 0.01$.

(E) Lats1/2 and Mst1/2 knockdown with 5nM siRNA in HaCaTs. Immunofluorescence analysis for Yap. Scale bars represent 20 μm.

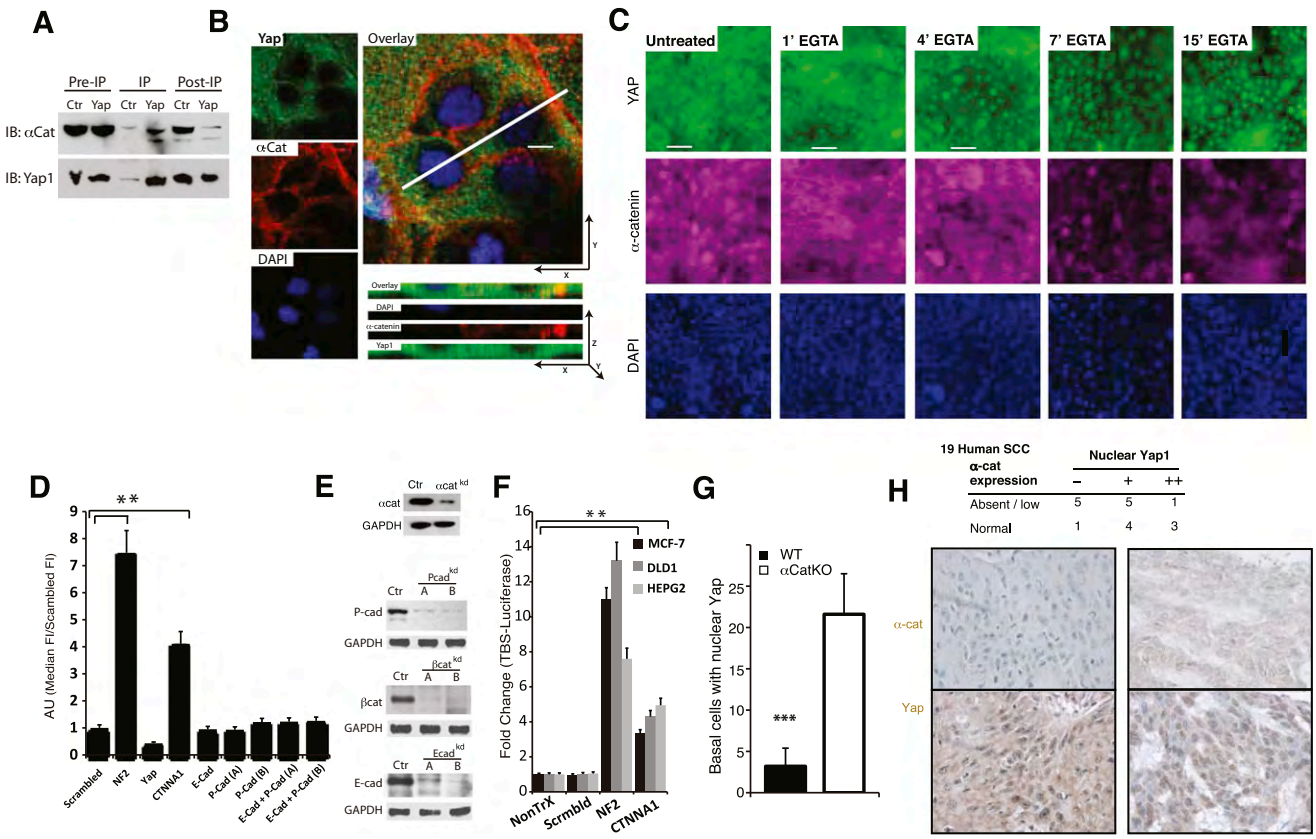

Figure S5. Localization and Expression of α-Catenin Suppresses TEAD-Based Reporters and Correlates with Nuclear Yap1 in Human Tumors, Related to Figure 5 and Figure 6

(A) Immunoprecipitation of Yap1 from primary keratinocytes isolated from newborn murine epidermis. Immunoreactive bands indicate the presence of α-catenin in the Yap1-immunoprecipitate as compared to an IgG control.

(B) Confocal microscopy of Yap1 and α-catenin in confluent primary human keratinocytes demonstrating the presence of Yap1 in α-catenin enriched sites at the cell membrane. Note also the presence of Yap1 in the cytoplasm and the absence of nuclear Yap1.

(C) Yap1 localization is responsive to EGTA treatment. High-density cultures of HaCaT cells were exposed to 4 mM EGTA for different periods of time. Cells were then fixed immediately after each indicated time point and stained for Yap1 and α-catenin. Note the rapid nuclear localization upon calcium chelation is evident after 1 min.

(D) Simultaneous knockdown of E-cadherin and P-cadherin has no effect on TBS-reporter activity. HaCaT cells were transfected with siRNAs targeted against E-cadherin as well as P-cadherin. NF2 and α-catenin siRNA knockdowns were utilized as positive controls. Note that no significant change was detected in TEAD-mediated reporter activity. Results represent mean of three experimental replicates ± standard deviation. $^{**}p < 0.01$.

(E) Western blot analyses of siRNA knockdown of α-catenin, P-cadherin, E-cadherin, and β-catenin. A and B represent two different siRNAs. GAPDH was used as a loading control.

(F) Activation of the TBS-reporter by α-catenin knockdown is conserved across various cell-type. MCF-7, DLD1 and HEPG2 cells were transfected with siRNAs against α-catenin and TBS-luciferase/Renilla reporter plasmids. Results represent mean of three experimental replicates ± standard deviation. $^{**}p < 0.01$.

(G) Quantification of predominantly nuclear Yap1 localization in the basal layer of α-cat$^{-/-}$ mouse epidermis.

Results represent mean of 2 experimental replicates (6 fields of view per animal) ± standard deviation. $^{***}p < 0.001$.

(H) α-catenin levels correlate with Yap1 nuclear localization in human tumors. Immunohistochemical analysis of α-catenin (α-cat) and Yap1 in 19 human squamos cell carcinomas (SCCs) of different grades. Table summarizes extent of Yap1 nuclear localization (-, not detected; +, some positivity, ++ high positivity) in SCCs divided in two sub-groups: those expressing no or low levels of α-cat and those expressing relatively normal amounts. Two examples of human SCC expressing low α-cat levels displaying + (left) or ++ (right) positivity for nuclear Yap1.

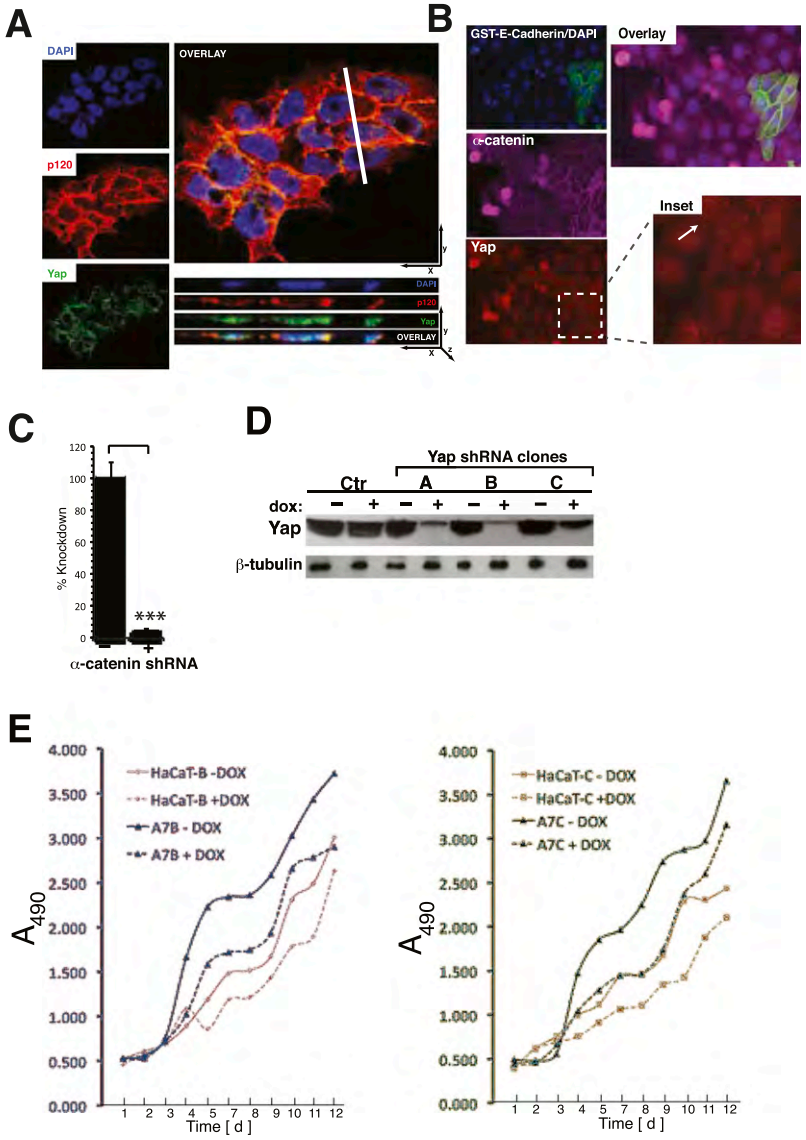

Figure S6. Yap1 Mediates the Growth-Suppressive Functions of α-Catenin, Related to Figure 6

(A) Yap1 and p120-catenin co-localize at adherens junctions. HaCaT cells seeded at low cell-density, ectopically expressing α-catenin, were analyzed by confocal microscopy for localization of Yap1. Immunocytochemistry and z-stack analysis using p120-catenin (red) and Yap1 (green) antibodies reveals co-localization at sites of cell-cell contact. Nuclear Yap1 is also present, reminiscent of Yap1 localization under low cell-density conditions.

(B) E-cadherin does not promote the re-localization of Yap1 to the membrane. Confocal microscopy studies evaluating immunofluorescence stains for Yap1 (red) in low-density HaCaT cells ectopically expressing GST E-cadherin (green) do not show Yap1 sequestration to sites of cell-cell contact (arrow).

(C) Quantitative RT-PCR analysis (right) of α-catenin levels in a HaCaT keratinocyte cell line stably expressing a shRNA against α-catenin. Results represent mean of three experimental replicates ± standard deviation. ***$p < 0.001$.

(D) Immunoblot analysis of three independent cell lines expressing inducible shRNAs against Yap1. Note the reduced levels of Yap1 protein after doxycycline induction.

(E) MTS proliferation assays reveal that the knockdown of Yap1 with three independent shRNAs (B and C, data from A shRNA shown in Figure 4g) rescues the hyperproliferative phenotype caused by the depletion of α-catenin. Dox-inducible knockdown of Yap1 in HaCaT cells (HaCaTB + dox, HaCaTC + dox) results in decreased cell proliferation compared to the uninduced control (HaCaTB – dox, HaCaTC – dox) as measured by the absorbance at 490 nm. Stable-knockdown of α-catenin in (A7B - dox, A7C - dox) promotes hyperproliferation, whereas Dox-induced knockdown of Yap1 (A7B + dox, A7C + dox) decreases the cell proliferation rate.

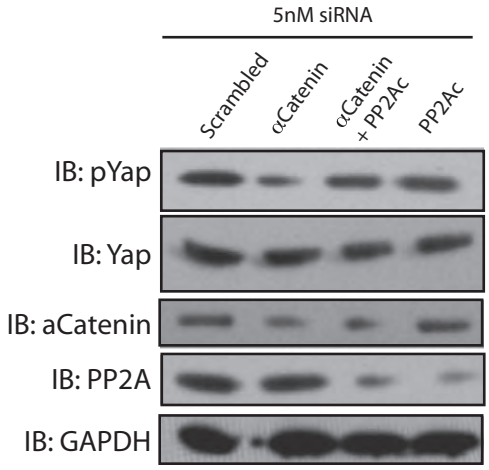

Figure S7. siRNA Knockdown of PP2Ac and α-Catenin Restores Yap1 Phosphorylation Levels, Related to Figure 7

Western blot analysis on HaCaT cells transfected with 5 nM siRNA against a-catenin, PP2Ac, and α-catenin/PP2Ac. Phosphorylation levels of Yap1 were measured using a pYap antibody that specifically recognizes phosphorylation at S127.

3.1 SCC-like Tumor Formation upon *Yap1* activation is reversible

In order to assess the long-term effects of *Yap1* activation, newborn Tg and control (CTR) skin was grafted onto Nude mouse recipients followed by Dox administration. Epidermal thickening, hyperkeratosis, and stunted hair growth were evident in Tg-grafted mice shortly after skin transplantation. Beginning at 20 days, Tg grafts developed large tumor-like masses that subsequently ulcerated (MANUSCRIPT I, Figure 2A). The morphological perturbations resembled those of squamous cell carcinoma (SCC) *in situ* in human (MANUSCRIPT I, Figure 2B). When Dox administration and, therefore, Tg activation was stopped six weeks after transplantation, the SCC-like tumors regressed and hair growth was initiated again, as shown in Figure 8.

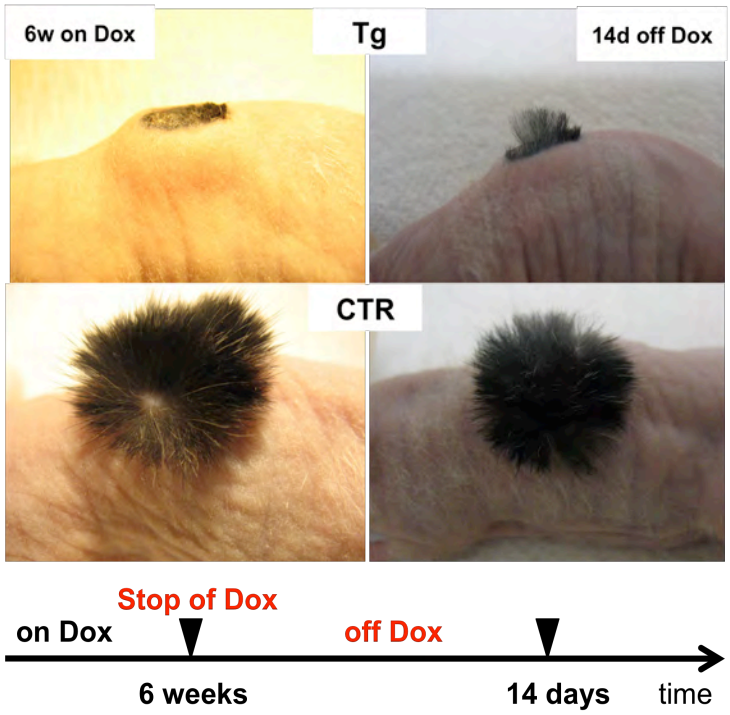

Figure 8: SCC-like tumor formation upon *Yap1* activation is reversible.
Left: Newborn CTR and Tg mouse skin were grafted onto Nude mice, which were subsequently administered doxycyclin (DOX) for six weeks. Right: Dox induction was stopped six weeks after transplantation and CTR and Tg grafts were monitored for 14 days.

3.2 *Yap1* cKO skin grafts can be sustained

Loss of *Yap1* in the K14-expressing progeny of the epidermis starting at E13.5 led to a severe skin defect in embryos at E18.5 and embryos died shortly after birth (MANUSCRIPT I, Figure 3A and B). Histological analysis revealed a noticeably thinner skin in *Yap1*-cKO embryos compared with the CTR (MANUSCRIPT I, Figure 3C). To assess the long-term effects of *Yap1*- deficiency in the epidermis, we isolated the back skin of CTR and *Yap1*-cKO embryos and grafted it onto Nude mice. *Yap1*-cKO full-thickness skin grafts could be sustained for seven weeks and no macroscopic abnormalities were observed, except a decrease in hair density (Figure 9).

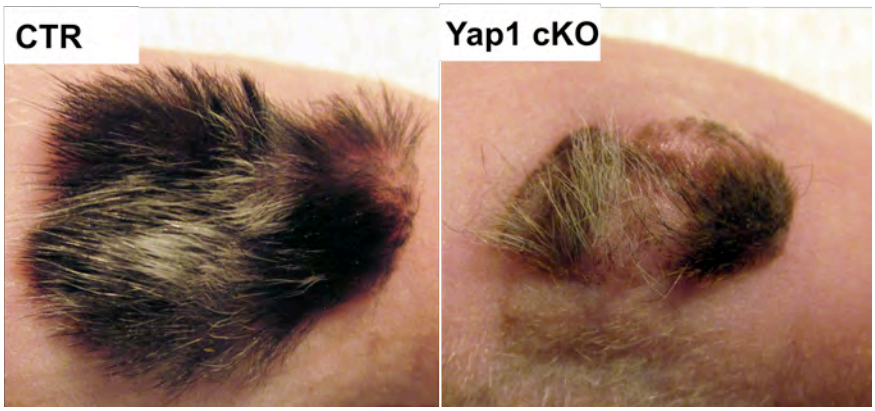

Figure 9: *Yap1* deficient skin grafts can be maintained under homeostatic conditions.
Gross morphology of control (CTR) and *Yap1* cKO grafts 7 weeks after transplantation.

4. Discussion

It is becoming evident that the novel Hippo signaling pathway plays a crucial role for the regulation of mammalian organ growth. However, most of the *in vivo* results available were obtained studying the liver and little is known about its function in the skin. Organ growth necessitates complex and precisely regulated mechanisms involving the tissue resident stem cells. Interestingly, the Camargo laboratory showed expression of the downstream effector of the Hippo pathway, YAP, to be localized in compartments of the intestine and epidermis, that comprise the tissue resident stem cells (Camargo et al., 2007).

Further results implicating Hippo signaling in epidermal biology came from a study showing that the deletion of *Sav1* led to hyperproliferation in the epidermis (Lee et al., 2008). Interestingly, only few factors with an essential function for continual stem cell proliferation *in vivo* are known in the epidermis, such as the transcription factors p63 and TCF3/TCF4 (Senoo et al., 2007; Nguyen et al., 2009). Unraveling the molecular mechanisms of epidermal stem cell proliferation is of great relevance to enable the development of treatments for skin diseases with a proliferation defect, such as psoriasis. Furthermore, knowledge about these signals could elucidate the origins of epidermal cancers, since tissue growth and tumorigenesis share common cellular signaling mechanisms. The focus of this thesis is, therefore, the investigation of the *in vivo* function of the Hippo pathway in the epidermis with emphasis on epidermal stem cell proliferation.

4.1 YAP as a Critical Regulator of Epidermal Stem Cell Proliferation

Using gain- and loss-of-function mouse models, it was possible to provide genetic evidence in this work that the Hippo pathway components YAP1 and the TEAD transcription factors are crucial for the long-term proliferation capacity of epidermal stem cells. Predominantly nuclear YAP1-S127A (Tg) expression in the adult epidermis resulted in an expansion of epidermal progenitors, marked by K5- and p63-expression, as well as in an impairment in

differentiation, illustrated by the lack of differentiation markers K10 and Loricrin (MANUSCRIPT I, Figure 1 and S1). These results were confirmed for newborn epidermis by another study, in which Tg expression occurred during embryonic development (Zhang et al., 2011). Moreover, an additional study found that expression of the predominantly nuclear and transcriptionally active YAP2-5SA-ΔC under the K5 promoter led to hyperproliferation in the postnatal mouse epidermis (Beverdam et al., 2012). Together these findings demonstrate that increased active YAP levels during embryonic development as well as in the postnatal murine epidermis induce progenitor proliferation.

To study the corresponding loss of *Yap1*, we generated a conditional floxed allele of *Yap1* by gene targeting (cKO) (MANUSCRIPT I, Figure S2A). Embryos lacking YAP expression in the epidermis displayed severe epidermal defects at E18.5, with the skin being noticeably thinner overall and even lacking completely on the distal limbs, the ears and the eyes (MANUSCRIPT I, Figure 3A-C). The epidermal morphology was compromised, showing a hypoplastic basal layer with a decreased proliferation rate (MANUSCRIPT I, Figure 3E and 3F). We conclude from these experiments that YAP is necessary for epidermal proliferation during development.

As demonstrated, the proliferation rate in the epidermis of the limbs at E18.5 was more than three-fold increased compared to the epidermis of the back (MANUSCRIPT I, Figure S2E). This reflects the increased demand for proliferation in the epidermis of these fast-growing parts of the body. Upon deletion of *Yap1*, this proliferation demand cannot be met, resulting in a more pronounced phenotype in the area of the limbs, compared with the epidermis of the back. When *Yap1* cKO back skin was transplanted onto Nude mice, however, grafts were sustained and showed no major macroscopically visible defects other than a reduction in hair growth (Figure 9). Results by other studies show that YAP expression in the epidermal basal layer decreases with age and is, moreover, localized mainly in the cytosol of postnatal basal cells (Zhang et al., 2011; Beverdam et al., 2012). Together with our findings, this implies that YAP at endogenous levels is more important for epidermal proliferation during development, than under homeostatic conditions in the postnatal epidermis.

YAP can interact with a variety of transcription factors, and even though Hippo signaling activity seems primarily to be transmitted through the TEAD transcription factors, it is

unknown if tissue specificity exists in this regard. To address whether TEAD transcription factors are important for epidermal biology, we generated a mouse line expressing a mutated YAP1-S79A that cannot interact with the TEAD transcription factors (MANUSCRIPT I, Figure S3). In combination with the floxed *Yap1* (cKO) allele, the remaining functional YAP1 protein carried the S79A mutation. With this experiment we were able to demonstrate that without TEAD interaction, embryos at E18.5 displayed epidermal defects reminiscent of the phenotype observed for the *Yap1* cKO (MANUSCRIPT I, Figure 4D). We conclude from these results that YAP1 mediates its function in epidermal proliferation in a complex with the TEAD transcription factors. These results also imply that the target genes mainly responsible for mediating the pro-proliferative effect of the YAP1/TEAD-complex should contain TEAD-binding sites near or in their promoter region. ChIP-Seq analysis in keratinocytes could identify additional target genes for epidermal Hippo signaling.

4.2 YAP and Skin Cancers

In the liver, YAP activation was found to lead to hepatocellular carcinoma formation (Dong et al., 2007). Elevated YAP levels are furthermore found in various human cancers. In this work, YAP1 was identified as a novel essential factor for epidermal stem and progenitor cell proliferation. In order to assess long-term effects of Tg expression, we performed full-thickness skin grafts onto immunocompromised Nude mice. The continuous thickening of the epidermis was accompanied by hyperkeratosis and stunted hair-growth. In this work, Tg expression was shown to lead to the development of squamous cell carcinoma (SCC) like tumors (MANUSCRIPT I, Figure 2). Remarkably, these phenotypes were reversible and tumors regressed in size and even hair growth was initiated again, once Tg induction was stopped (Figure 8). The results show that the precise regulation of YAP1 activity is essential for tumor suppression in the epidermis. In their study, Zhang et al. additionally found human SCC and basal cell carcinoma (BCC) tissue to stain positive for nuclear YAP (Zhang et al., 2011). These findings combined suggest an activation of the Hippo pathway through YAP in epidermal cancers.

4.3 A Connection of α-Catenin and the Hippo Pathway

In recent years, it has become clear, that the AJ component α-catenin plays more than just a structural role. One study in particular implicated α -catenin in epidermal stem cell proliferation. The deletion of α-catenin in the epidermis, interestingly, led to a hyperproliferative phenotype reminiscent of the phenotype that we observed for Tg expression in the epidermis (Vasioukhin et al., 2001). Moreover, reduced α-catenin levels are found in a variety of human cancers (Shimoyama et al., 1992; Morton et al., 1993; Kadowaki et al., 1994; Matsui et al., 1994; Ewing et al., 1995; Bullions et al., 1997). Many of α -catenin's interaction partners are known and implicated in cell-cell adhesion, such as vincullin, α -actinin, formin, zonula occludens protein 1 (ZO1) and afadin (AF6) (Kobielak and Fuchs, 2004). The finding of our group, that α-catenin can also associate with YAP, the downstream effector of a pathway controlling organ growth, strengthens the emerging view of α-catenin as a signaling molecule with transcriptional influence similar to β -catenin. The results of our group show that α-catenin regulates YAP subcellular localization as well as activity, suggesting that the growth-suppressive effects of α-catenin in the epidermis might be mediated through inactivation of YAP.

Other studies previously demonstrated a direct interaction of α-catenin and Ajuba. Ajuba was recently identified as a Hippo pathway component that localizes to AJ and, similar to β -catenin, can also translocate to the nucleus (Kanungo et al., 2000; Marie et al., 2003; Thakur et al., 2010). This suggests additional connections between cell adhesion and transcriptional regulation, which will need to be explored further in the future. NF2, another component of the Hippo signaling pathway, has been demonstrated to be able to modulate epidermal development via an association with AJs, specifically α-catenin and the Par3 polarity complex (Gladden et al., 2010). Taken together, our and previously published results point to a scenario in which multiple signaling cues sensing cell density and determining cell positioning converge on Hippo signaling components and thereby coordinate organ growth.

4.4 Regulation of YAP Activity by 14-3-3 and PP2A

It is already known that 14-3-3 proteins participate in Hippo pathway regulation by binding phosphorylated YAP protein and thus influencing the subcellular localization and activity of YAP. The results obtained by our group extend this knowledge by showing that the 14-3-3 protein is crucial for the association of YAP with α-catenin. The isoform 14-3-3σ, expressed in stratified epithelia, has been implicated in the regulation of epidermal proliferation (Dellambra et al., 2000; Pellegrini et al., 2001). Interestingly, epidermal expression of a dominant-negative form of 14-3-3σ induces hyperproliferation, similarly observed for deletion of α-catenin or YAP activation (MANUSCRIPT I Figure 1; Vasioukhin et al., 2001; Herron et al., 2005; Li et al., 2005). These findings combined suggest a regulatory role for the complex of YAP, α-catenin and 14-3-3σ in epidermal stem cell proliferation.

One mechanism to regulate protein activity in the cell involves the competitive binding of 14-3-3 proteins to protein residues carrying a phosphate group, and the dephosphorylation of these residues by protein phosphatase PP2A (Chiang et al., 2003; Martin et al., 2008). The data obtained in our group show a preferential association of YAP and the catalytic subunit of PP2A (PP2Ac) in α -catenin-deficient keratinocytes (MANUSCRIPT I, Figure 7A). In addition, we observed that Hippo pathway activity, measured by the TBS-reporter, increased upon α-catenin knock down by siRNAs. This was significantly reduced when PP2A was knocked down simultaneously (MANUSCRIPT I, Figure 7C). Combined, these results imply PP2A as the phosphatase that dephosphorylates YAP, triggering its activation. Likely scenarios suggest a YAP/14-3-3 complex, that is either stabilized or protected from PP2A access by α-catenin binding.

4.5 Crowd Control and the Hippo Pathway

In mammals, the extracellular upstream signals that regulate the activity of the Hippo signaling pathway are unknown. The deletion of α-catenin induced massive overgrowth phenotypes in the skin and the developing brain, leading to the postulation that AJs could function as molecular biosensors of extracellular cues, such as cell density and positioning (Lien et al., 2006a; 2006b). The data obtained in our group support this hypothesis and

indicate that in the epidermis YAP participates in the molecular circuitry that senses cell density through interaction with α-catenin (MANUSCRIPT I, Figure 7). We postulate a model for the epidermis in which high cell density and differentiation lead to the inactivation of YAP. AJs and α-catenin serve as "crowd control" sensors in this scenario. In the differentiating, suprabasal layers of the epidermis YAP could get inactivated by the selective expression of 14-3-3σ. A low cellular density of the basal cells, as in a growing embryo or after wounding, would translate into nuclear YAP and proliferation.

4.6 YAP Regulates Cardiomyocyte Proliferation

The deletion of the core pathway component *Sav1* in the developing embryonic heart resulted in an increase in heart size or cardiomegaly (Heallen et al., 2011). Loss of *Lats1/2* and *Mst1/2* led to similar phenotypes, implicating the Hippo signaling pathway in the regulation of embryonic heart growth. Our collaborators in the Pu laboratory elucidate the function of YAP1 in the unique situation of heart growth (Appendix MANUSCRIPT II; Gise et al., 2012). Traditionally, heart growth is divided into two stages: embryonic growth, which is achieved through cardiomyocyte proliferation, and postnatal growth, which is thought to occur through cardiomyocyte hypertrophy (Li et al., 1996a). Von Gise et al. found that YAP1 is crucial for fetal cardiomyocyte proliferation, with deletion leading to lethal myocardial hypoplasia and decreased cardiomyocyte proliferation. Overall organ size and gross morphological anatomy were only marginally affected (Appendix MANUSCRIPT II, Figure 1). Correspondingly, YAP1 activation in the fetal heart increased cardiomyocyte proliferation, which was corroborated in another study (Appendix MANUSCRIPT II, Figure 4; Xin et al., 2011). Interestingly, *Yap1* deletion at postnatal day 3, when heart growth is beginning to be sustained through an increase in cell size rather than cell number, did not alter cardiomyocyte size (Appendix MANUSCRIPT II, Figure 2B). Therefore, in the heart YAP1 regulates embryonic cardiomyocyte proliferation and, hence, heart development, but it appears to be dispensable in postnatal hypertrophic growth.

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a myocardial disease that can cause arrhythmia, heart failure, and sudden death. ARVC often occurs in combination with defects in skin and hair (palmoplantar keratoderma and woolly hair), which is known as

Naxos syndrome (McKoy et al., 2000; Rapini et al., 2007). Interestingly, mutations of plakoglobin (also called γ-catenin) were identified in patients with Naxos syndrome (McKoy et al., 2000). Based on our findings that α-catenin regulates YAP1 activity, it is intriguing to speculate that plakoglobin might regulate YAP1 activity in a similar fashion. This could imply the involvement of YAP1 and Hippo signaling in ARVC and Naxos syndrome.

4.7 Tissue Specificity of the Hippo Pathway

Based on previous studies, it seems that the requirement for YAP during development, homeostasis and regeneration varies greatly in different organs. The loss of *Yap1* in the intestine had no obvious effects on development and homeostasis, but impaired adult regeneration (Cai et al., 2010; Barry et al., 2013). In the liver, defective biliary development was observed, but regeneration after partial hepatectomy was not impaired (Zhang et al., 2010b, Camargo unpublished data). The findings of this thesis reveal the importance of the YAP1-TEAD complex for epidermal proliferation during development. This essential function of YAP1 for self-renewal reminds of its function in human embryonic stem cells (Lian et al., 2010).

It is noteworthy that even though *in vivo* deletion of *Sav1* led to hyperproliferation in the epidermis, deletion of *Mst1/2* did not induce a likewise effect in our hands. We also showed with *in vitro* experiments that knock down of the *Mst1/2* transcript had no effect on YAP phosphorylation. This suggests the existence of other hitherto unidentified kinases besides MST1/2 to inactivate YAP in epidermal cells. Identification of a kinase that mediates YAP inactivation in keratinocytes will likely provide insights into the mechanisms that underlie SC maintenance, tissue expansion, and tumorigenesis.

It is becoming apparent that the Hippo signaling pathway is very diverse in mammals and shows tissue-specific alterations to the originally identified core kinase cascade. Previous work in *Mst1*$^{-/-}$ *Mst2*$^{fl/-}$ mouse embryo fibroblasts (MEFs) has demonstrated that MST1/2 are dispensable for the regulation of YAP activity, since YAP phosphorylation levels were unaltered upon retroviral Cre-excision (Zhou et al., 2009). The results go in line with our results in the human keratinocyte cell line HaCaT, where knockdown of the *Mst1/2* transcript

altered neither YAP phosphorylation levels nor its subcellular localization pattern, suggesting no influence of MST1/2 on YAP activity (MANUSCRIPT I, Figure S4). In the liver, however, MST1/2 were demonstrated to be necessary for YAP phosphorylation, and interestingly, in this tissue the other important core kinases LATS1/2 were expendable (Zhou et al., 2009).

The YAP homolog TAZ was shown to be equally regulated by the Hippo signaling cascade (Lei et al., 2008). During development YAP and TAZ vary in their functions. *Taz* deletion led mainly to defects in the kidney and lung, whereas loss of *Yap1* led to embryonic lethality at E8.5 (Morin-Kensicki et al., 2006; Hossain et al., 2007; Tian et al., 2007; Makita et al., 2008). Yet, their combined deletion resulted in embryonic lethality before the morula stage, suggesting compensatory effects to some extent (Nishioka et al., 2009). Currently, it is unknown whether YAP and TAZ are able to compensate for each other in adult tissues. Compensation by TAZ could explain the observed results that YAP at endogenous levels is less important during adult homeostasis of the epidermis. Moreover, compensatory effects by TAZ could account for the observation that YAP might not to be required for postnatal hypertrophic heart growth. Future research will have to examine the effects of a combined tissue-specific deletion of *Yap1* and *Taz* in the epidermis and the heart.

Recently, it was also shown that mechanical tension is able to influence YAP activation (Dupont et al., 2011; Wada et al., 2011; Zhao et al., 2012). Especially in light of our group's finding that the AJ component α-catenin is an upstream regulator of Hippo signaling in the epidermis, it is intriguing to speculate that the tissue-specific expression of factors relaying extracellular signals, such as cellular adhesion complexes and polarity complexes are involved in determining context dependent variations of the Hippo cascade.

4.8 Future Directions

Eyelid fusion occurs during mouse embryogenesis around E15.5 and has been shown to involve a plethora of signaling pathways such as EGF, TGFα, BMP, Shh, JNK and Wnt (Findlater et al., 1993; Luetteke et al., 1993; 1994; Andl et al., 2004; Weston et al., 2004;

Mine et al., 2005; Tao et al., 2005; Gage et al., 2008; Takatori et al., 2008). It is interesting to note that the open-eye phenotype observed in this work upon deletion of *Yap1* was also observed when Zhang et al. expressed constitutively active YAP1-S127A in embryos (MANUSCRIPT I, Figure 3; Zhang et al., 2011). Hippo signaling seemingly plays a part in this process and precisely regulated YAP levels might be needed for proper eyelid fusion to occur. Future investigations could explore how exactly the Hippo pathway participates in this process.

Identification of the downstream targets of Hippo signaling in the skin will be of immense interest for a better characterization of the pathway. Zhang et al. identified Cyr61 as one of the major downstream targets in keratinocytes *in vitro* and *in vivo*, whereas CTGF expression was upregulated upon *Yap1* activation only *in vivo* (Zhang et al., 2011), suggesting a potentially differential expression for the two genes. The development of an *in vivo* Cyr61 and CTGF-reporter system could reveal the localization of Cyr61 and CTGF expression within the epidermis, which could represent epidermal stem and/or progenitor cells.

The current model for epidermal homeostasis involves the quiescent stem cells and TA cells, but markers distinguishing between the two populations in the interfollicular epidermis are poorly defined. It is noteworthy that the K14-promoter, currently used for manipulation of epidermal progenitors *in vivo* and also used in this thesis, is active throughout the basal cell layer of the epidermis. Therefore, it is not possible at this moment in the field to discern specific effects on SCs and TA cells of the interfollicular epidermis. It is possible that YAP might be important for either one of the two populations or both. Utilizing Cyr61 and CTGF-reporters could provide means to address this question. One initial experiment could be the isolation of reporter-expressing keratinocytes and analysis of their proliferative capacity by *in vitro* proliferation assays according to their potential to give rise to holoclones (SCs) and/or paraclones (TA). The existence of meroclones, intermediates between holoclones and paraclones in terms of their appearance and their reproductive behavior, could imply the existence of an epidermal SC that gives rise to a continuum of cell populations with a progressively decreasing capability to proliferate and self-renew (Barrandon and Green, 1987). It is to be kept in mind that in general SCs and TA cells may not necessarily be two completely distinct populations, but instead there might exist a gradient in the cell's capacity for self-renewal and differentiation (Potten and Loeffler, 1990). Better characterization of SC

and TA cells is necessary to elucidate the molecular basis for diseases such as psoriasis, where epidermal homeostasis is altered. Some studies suggest that TA and not SCs carry the intrinsic defect leading to the development of the lesions (Franssen et al., 2005; Grabe and Neuber, 2007).

Also currently lacking in the Hippo field is a *in vivo* TEAD-binding sites (TBS)- containing reporter, similar to the TOPflash reporter, widely used to monitor Wnt-pathway activity. Generating an *in vivo* reporter, analogously to the *in vitro* TBS-reporter used in our laboratory, that measures Hippo signaling activity, could aid the characterization of stem cells. It serves as a marker and would enable the isolation and characterization of cells with varying Hippo activity, and thus a comprehensive profiling. Moreover, it would help to identify which pathway components are essential for Hippo signaling activation in the different organs of the body.

During epidermal development, the stem cell's mitotic spindle shifts and is oriented perpendicular to the BM. These perpendicular cell divisions, where one cell loses contact with the BM, ensure the establishment of the differentiated suprabasal layers and thus the growth of the epidermis. In their study, Zhang et al. also found that YAP expression decreases with age. They suggested a correlation between nuclear YAP and proliferation potential of epidermal progenitors (Zhang et al., 2011). Interestingly, previous research suggested that also the proportion of perpendicular asymmetric cell divisions in the epidermis decreases in the adult (Lechler and Fuchs, 2005). Moreover, using RNA interference i*n vivo* to impair asymmetric cell divisions led to defects in epidermal barrier formation and stratification (Williams et al., 2011). It would be interesting to investigate if expression of the Tg could rescue these described epidermal defects. The phenotype obtained in the epidermis upon deletion of α-catenin and activation of *Yap1* is very similar. Intriguingly, Lechler et al. found randomization of spindle alignment and misoriented cell divisions in epidermis from α-catenin null mice (Lechler and Fuchs, 2005). Therefore, one may speculate that precisely regulated nuclear YAP levels could be necessary during cell division for proper spindle orientation. Future investigations could examine a potential function of YAP for asymmetric cell division in the epidermis.

Our results suggest YAP as a possible therapeutic target. Elucidating the Hippo signaling pathway in the epidermis further will be crucial for our understanding of skin diseases, associated with aberrant proliferation, such as psoriasis and epidermal cancers.

5. Summary

The tight regulation of organ size is a prerequisite of unperturbed development of complex organisms. The molecular mechanisms determining and regulating the size of entire organisms and organs are poorly understood. The actual growth process of an organ requires stem cells, which integrate signals from pathways involved in the initiation or termination of pro-proliferative programs. Recently, the Hippo signaling pathway, with its transcriptional effector YAP1, has been discovered as a critical regulator of both, organ size control and tumorigenesis, two processes with common cellular signaling mechanisms. Therefore, it is of interest to determine, how precisely Hippo signaling regulates progenitor and stem cell proliferation. The mammalian epidermis is a rapidly regenerating epithelial tissue, whose maintenance depends on the self-renewing ability of epidermal stem cells, which reside in the basal layer. Epidermal stem cells are essential for skin homeostasis and regeneration during wound repair. Interestingly, the epidermal deletion of *Sav1* (*WW45*), a core component of the Hippo signaling protein cascade, was shown to increase the progenitor population in the basal layer of the epidermis. In this work, we provide further evidence, that the Hippo pathway, and its nuclear effector YAP1 are important for the regulation of stem cell proliferation in the epidermis.

Using gain- and loss-of-function studies, we show that activation of *Yap1* in the basal layer leads to a hyper-thickened epidermis, due to an expansion of the epidermal stem cell compartment, and a decrease of the suprabasal differentiated cell layers. Moreover, we demonstrate that long-term constitutive activation of *Yap1* in the epidermis leads to the formation of squamous cell carcinoma-like tumors. Deletion of *Yap1* (cKO) in the basal layer of the epidermis, on the other hand, causes a severe skin defect with an abnormally thin epidermis due to decreased epidermal proliferation. Further, we show, that YAP1 regulates epidermal proliferation through interaction with the TEAD transcription factors. The YAP1-S79A mutation has been shown to impair the interaction with the TEAD transcription factors. Mice expressing solely YAP1-S79A in the basal layer phenotypically resemble *Yap1* cKO mice. Loss of the MST1/2 kinases, which are considered core components of the Hippo pathway, was previously shown to lead to hepatomegaly in the liver. In this work we show

that loss of the MST1/2 kinases in the epidermis does not lead to a hyperproliferative phenotype as observed for epidermal *Yap1* activation. This observation suggests that YAP1 might be regulated by other mechanisms in the epidermis. α-catenin is implicated in tumor suppression and cell density sensing in the skin. Our group determined an association of YAP1 and α-catenin. We identify α-catenin as an upstream negative regulator of YAP1. α-catenin controls YAP1 activity and phosphorylation by modulating its interaction with 14-3-3 and the PP2A phosphatase and, therefore, regulates the proliferative function of YAP1 in keratinocytes. Together, these data identify YAP1 as a determinant of the proliferative capacity of epidermal stem cells and as an important effector of a ‘‘crowd control’’ molecular circuitry in mammalian skin.

In collaboration with the Pu laboratory we further elucidate, that activation *of Yap1* in the fetal heart induces cardiomyocyte proliferation. Correspondingly, *Yap1* deletion causes lethal myocardial hypoplasia and impaired cardiomyocyte proliferation.

The findings presented in this thesis show that in the epidermis and the heart, YAP1 is essential for proliferation and normal tissue development. Moreover, this work demonstrates the diversity of the Hippo signaling machinery in different tissues, showing that in the epidermis the core kinases MST1/2 do not regulate YAP1-mediated proliferation. Thus, it provides novel insights about the Hippo signaling pathway and may ultimately contribute to a better understanding of this pathway in both, normal physiological and disease conditions.

6. Zusammenfassung

Die normale Entwicklung von komplexen Organismen setzt eine präzise Regulation der Organgröße vorraus. Jedoch sind die molekularen Mechanismen, welche die Größe von gesamten Organismen und einzelnen Organen bestimmen, weitgehend unbekannt. Stammzellen sind für den Wachstumsprozess eines Organs essentiell. Sie verarbeiten Signale, die pro-proliferative Expressionsprogramme aktivieren oder beenden. Es wurde kürzlich entdeckt, dass der Hippo Signalweg, einschliesslich seines transkriptionellen Coaktivators YAP1, sowohl bei der Regulation der Organgröße als auch in der Karzinogenese involviert ist, zwei Prozessen mit gemeinsamen zellulären Signalmechanismen. Daher ist es von großem Interesse herauszufinden, wie genau der Hippo Signalweg die Progenitor- und Stammzellproliferation steuert.

Die Epidermis ist ein sich fortlaufend erneuerndes Epithelgewebe, welches für seinen Erhalt Stammzellen mit der Fähigkeit zur Selbsterneuerung benötigt. Epidermale Stammzellen sind sowohl für die Homöostase der Haut, als auch für die Wundheilung essentiell. Interessanterweise wurde gezeigt, dass in der Epidermis der Verlust von *Sav1* (*WW45*), einem Kernbestandteil der Hippo Signalkaskade, zu einer Vermehrung der Progenitorpopulation in der Basalschicht führt. Die Ergebnisse dieser Arbeit zeigen, dass der Hippo Signalweg und im Speziellen sein Effektor YAP1, wichtig für die Regulation der Stammzellproliferation in der Epidermis sind.

Anhand von Gain-of function- und Loss-of-function-Mausmodellen, zeigen wir, dass die Aktivierung von *Yap1* in der Basalschicht der Epidermis eine Hautverdickung basierend auf der Vermehrung der epidermalen Stammzellen hervorruft. Zusätzlich kommt es zu einem Verlust an suprabasalen differenzierten Schichten der Epidermis. Eine andauernde konstitutive Aktivierung von *Yap1* in der Epidermis ruft zudem die Bildung von Plattenepithelkarzinom (SCC)-ähnlichen Tumoren hervor. Im Gegensatz dazu verursacht der Verlust von *Yap1* (cKO) in der Basalschicht der Epidermis einen schweren Defekt der Haut. Aufgrund einer verringerten Proliferationsrate kommt es zu der Bildung einer ungewöhnlich dünnen Epidermis. Wir zeigen, dass der Co-Aktivator YAP1 die Proliferation der Epidermis durch eine Interaktion mit den TEAD Transkriptionsfaktoren reguliert. Es wurde für die

YAP1-S79A Mutationsform gezeigt, dass eine Interaktion mit den TEAD Transkriptionsfaktoren nicht mehr stattfinden kann. Wir zeigen, dass der Phenotyp von Mäusen, die lediglich YAP1-S79A in der Basalschicht der Epidermis exprimieren, dem der *Yap*1 cKO Mäuse gleicht und einen Proliferationsdefekt aufweist. Zuvor wurde in der Leber gezeigt, dass der Verlust der MST1/2 Kinasen, die zu den zentralen Kinasen des Signalwegs gehören, zu Hepatomegalie führt. Hier präsentieren wir Ergebnisse, die belegen, dass der Verlust der MST1/2 Kinasen in der Epidermis nicht zu dem durch epidermale *Yap1* Aktivierung hervorgerufenen hyperproliferativen Phenotypen führt.

Diese Ergebnisse legen nahe, dass YAP1 in der Epidermis durch andere Mechanismen reguliert wird. Tatsächlich stellt unsere Arbeitsgruppe eine Interaktion zwischen YAP1 und α-Catenin fest. α-Catenin fungiert als Tumorsuppressor und gibt Informationen über Zelldichte weiter. Wir identifizieren α-Catenin als negativen Regulator von YAP1. α-Catenin reguliert dabei die Aktivität und die Phosphorylierung von YAP1, indem es die Interaktion von YAP1 mit den 14-3-3 Proteinen und der Phosphatase PP2A moduliert. Hierdurch reguliert α-Catenin die proliferativen Eigenschaften von YAP1 in Keratinozyten. Zusammengefasst identifizieren diese Ergebnisse YAP1 als einen entscheidenden Faktor der proliferativen Eigenschaften von epidermalen Stammzellen, der somit bedeutend für die Homöostase der Haut ist und auch bei Krankheitsprozessen wie Krebs eine entscheidende Rolle spielen könnte.

In Zusammenarbeit mit dem Pu Labor zeigen wir zudem, dass die Aktivierung von *Yap1* im embryonalen Herzen zu einer vermehrten Proliferation der Kardiomyozyten führt. Entsprechend verursacht der Verlust von *Yap1* eine lethale Hypoplasie des Myokards und beeinträchtigt die Proliferation der Kardiomyozyten.

Die Ergebnisse dieser Arbeit in Epidermis und Herz demonstrieren die Bedeutung von YAP1 für zelluläre Proliferation und normale Gewebeentwicklung. Darüberhinaus gelingt es uns auch die gewebespezifische Vielfalt des Hippo Signalweges weiter aufzuschlüsseln. Wir zeigen, dass die Kinasen MST1/2 die YAP1-vermittelte Proliferation in der Epidermis nicht regulieren. Die Ergebnisse liefern neue Erkenntnisse über den Hippo Signalweg und tragen zu einem besseren Verständnis des Signalwegs in physiologischen und pathologischen Zuständen bei.

7. Abbreviation List

AJ – adherens junction

BM – basement membrane

cKO – conditional *Yap1* knockout

Cyr61 – cystein rich protein

CTGF – connective tissue growth factor

DOX – doxycyclin

ECM – extracellular matrix

EPU – epidermal proliferative unit

Ex – Expanded

HF – hair follicle

Hpo – Hippo

IFE – interfollicular epidermis

IRS – inner root sheath

K – keratin

LRC – label retaining cells

Mats – Mob as tumor suppressor

Mer – Merlin

ORS – outer root sheath

PP2A – protein phosphatase 2A

rtTA – reverse tetracycline transactivator protein

SC – stem cell

SG – sebaceous gland

TA – transit amplifying (TA cells)

TEAD – TEA-Domain transcription factor

Tg – transgene Yap1-S127A

TJ – tight junction

Wts – Warts

Yap – Yes-associated protein

Yki – Yorkie

8. Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die vorliegende Dissertation selbstständig verfasst, die für diese Arbeit benutzten Hilfsmittel genannt und die Ergebnisse anderer klar gekennzeichnet habe.

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Appendix

The Heart

The mammalian heart is a four-chambered muscular organ with the vital function to ensure continuous and lifelong circulation of blood throughout the body. During development, the heart is the first organ to be formed. The accurate growth to its final size and particular shape is a complex process that requires precise regulatory mechanisms, and involves different muscle and non-muscle cell populations. These cells arise from multipotent progenitor cells of the first and second heart field (Buckingham et al., 2005; Martin-Puig et al., 2008). The pathways responsible for the precisely coordinated growth of the heart are only partially understood.

Congenital heart defects and cardiac malformations represent one of the major birth defects in newborn children. In adults cardiovascular diseases (CVD) remain the leading cause of death worldwide (*World Health Statistics 2012* Geneva: WHO). A deficiency in heart size and muscularisation, like in ventricular noncompaction or arrhythmogenic right ventricular cardiomyopathy, cannot ensure sufficient cardiac output, whereas overgrowth phenotypes, as in hypertrophic cardiomyopathies equally pertub physiological heart function. These examples demonstrate, that organ size control is crucial for heart development and function. Traditionally, the growth of the heart during embryogenesis is considered to be sustained through cardiomyocyte proliferation, whereas the postnatally occurring growth is attributed mainly to hypertrophy of cardiomyocytes (Li et al., 1996b).

In this paradigm, the heart is considered a postmitotic organ, with postnatal growth occurring through increase in cell size rather than in cell number. Cardiac hypertrophy occurs not only in response to physiological stimuli such as exercise but also as a result of pathologic stimuli such as abnormal stress. Hence it is of therapeutic interest to unravel the underlying molecular mechanisms. Interestingly, the conventional view of the heart as a rather static organ is currently challenged. Several studies indicate that the heart is a self-renewing organ after all,

containing cardiomyocyte-like cells which undergo mitosis, even after birth (Potten, 1981; Beltrami et al., 2001; Anversa et al., 2006).

The Hippo signaling pathway has become known in recent years as a key regulator of organ size. Interestingly, one study demonstrated that the loss of the core pathway component *Sav1* in the developing embryonic heart resulted in an increase in heart size or cardiomegaly (Heallen et al., 2011). And also the loss of *Lats1/2* and *Mst1/2* lead to similar phenotypes, implicating the Hippo signaling pathway in the regulation of embryonic heart growth.

MANUSCRIPT II

YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy.

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Experimental Contribution

I contributed to the publication "YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy" intellectually and by generating the *Yap1* conditional knockout (cKO) as well as the *Yap1*-S79A mouse.

The original article is attached to the end of this thesis and online available at:
http://www.pnas.org/content/early/2012/01/24/1116136109

YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy

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Heart growth is tightly controlled so that the heart reaches a predetermined size. Fetal heart growth occurs through cardiomyocyte proliferation, whereas postnatal heart growth involves primarily physiological cardiomyocyte hypertrophy. The Hippo kinase cascade is an important regulator of organ growth. A major target of this kinase cascade is YAP1, a transcriptional coactivator that is inactivated by Hippo kinase activity. Here, we used both genetic gain and loss of *Yap1* function to investigate its role in regulating proliferative and physiologic hypertrophic heart growth. Fetal *Yap1* inactivation caused marked, lethal myocardial hypoplasia and decreased cardiomyocyte proliferation, whereas fetal activation of YAP1 stimulated cardiomyocyte proliferation. Enhanced proliferation was particularly dramatic in trabecular cardiomyocytes that normally exit from the cell cycle. Remarkably, YAP1 activation was sufficient to stimulate proliferation of postnatal cardiomyocytes, both in culture and in the intact heart. A dominant negative peptide that blocked YAP1 binding to TEAD transcription factors inhibited YAP1 proliferative activity, indicating that this activity requires YAP1–TEAD interaction. Although Yap1 was a critical regulator of cardiomyocyte proliferation, it did not influence physiological hypertrophic growth of cardiomyocytes, because postnatal *Yap1* gain or loss of function did not significantly alter cardiomyocyte size. These studies demonstrate that *Yap1* is a crucial regulator of cardiomyocyte proliferation, cardiac morphogenesis, and myocardial trabeculation. Activation of *Yap1* in postnatal cardiomyocytes may be a useful strategy to stimulate cardiomyocyte expansion in therapeutic myocardial regeneration.

heart development | physiological hypertrophy

Between the early heart tube stage and adulthood, the murine heart increases by >300-fold in mass (Fig. S1) (1). The ~18-fold increase in mass achieved during fetal life occurs mainly through cardiomyocyte proliferation, whereas the remaining ~18-fold growth achieved postnatally largely involves increased cardiomyocyte size (physiological hypertrophy), plus expansion of nonmyocyte populations. Growth of the heart is precisely regulated so that there is little variability in the final size of the adult heart. Derangements of these regulatory pathways likely contribute to congenital heart malformations, the leading form of major birth defect. Moreover, understanding of these pathways will be highly relevant for regenerative approaches to postnatal heart disease.

The Hippo signaling cascade was discovered in *Drosophila* as a potent mechanism that regulates cell proliferation and organ size (2, 3). The core kinases of this signaling pathway, Hippo (Hpo) and Warts (Wts), and their regulatory subunits Salvador (Sav) and Mats, phosphorylate Yorkie (Yki) (4–7). In the absence of phosphorylation by the Hpo kinases cascade, Yki coactivates transcription in conjunction with specific transcription factors, such as the TEAD family transcription factor Scalloped (Sd), stimulating organ growth by increasing cell proliferation and reducing apoptosis (8, 9). Incompletely understood upstream molecular signals activate the Hpo kinase cascade, leading to Yki phosphorylation, nuclear export, and reduced organ growth. The core of the Hippo pathway is highly conserved in mammals, where *Mst1/2*, *Sav1*, *Lats1/2*, *Yap1*, and *Tead1-4* are the orthologs of Hpo, Sav, Wts, Yki, and Sd, respectively (3). *Yap1* gain and loss of function in liver and skin suggest that *Yap1* plays an important role in mammalian organ size regulation, as in *Drosophila* (10–12).

In this study, we investigated the function of *Yap1* in regulating heart growth. *Yap1* loss of function impaired cardiomyocyte proliferation and caused lethal myocardial hypoplasia. Meanwhile, *Yap1* gain of function enhanced cell-cycle activity in cardiomyocytes both in vitro and in intact fetal and infant hearts. However, *Yap1* gain and loss of function did not substantially alter cell size, indicating that *Yap1* influences organ size primarily by regulating proliferation. Collectively, our data demonstrate that *Yap1* is a powerful regulator of cardiomyocyte proliferation.

Results

YAP1 Expression in Fetal and Postnatal Heart. We measured YAP1 expression at different stages of heart development. YAP1 protein was robustly detected in neonatal and juvenile mouse heart and declined with age, so that it was nearly undetectable by 12 wk of age (Fig. S2*A*). To distinguish expression in myocytes compared with nonmyocytes, we dissociated fetal, neonatal, and adult hearts and separated cells into myocyte and nonmyocyte fractions. By Western blotting, YAP1 was detected in both myocytes and nonmyocytes, with predominantly cardiomyocyte expression in fetal heart (Fig. S2*B*). In neonatal and 1.5-mo-old heart, expression levels in cardiomyocytes and nonmyocytes were comparable (Fig. S2*B*).

Fetal Cardiomyocyte-Restricted Loss of *Yap1* Causes Lethal Cardiac Hypoplasia. To test the hypothesis that *Yap1* is required for fetal cardiomyocyte proliferation, we inactivated a conditional *Yap1* allele ($Yap1^{flox}$) specifically in cardiomyocytes early in heart development using Tnnt2–Cre (13). To measure the extent of *Yap1* inactivation, we used FACS to isolate pure populations of cardiomyocytes from $Yap1^{fl/+}$::Tnnt2–Cre::$Rosa26^{mTmG/+}$ (*Yap1* heterozygous control) or $Yap1^{fl/fl}$::Tnnt2–Cre::$Rosa26^{mTmG/+}$

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The authors declare no conflict of interest.

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(*Yap1* mutant) heart at embryonic day (E) 12.5. In these embryos, Tnnt2–Cre activated membrane-localized GFP (mGFP) from the Cre-activated Rosa26^{mTmG} reporter and inactivated the conditional *Yap1* allele specifically in cardiomyocytes. By quantitative RT-PCR (qRT-PCR), *Yap1* transcripts were depleted by >95% at E12.5 in mutant compared with heterozygous control cardiomyocytes (Fig. 1*A*), confirming effective gene inactivation.

Yap1$^{fl/fl}$::Tnnt2–Cre (abbreviated Yap1^{Tnnt2}) embryos were present at a sub-Mendelian ratio by E12.5 and were not recovered beyond E16.5. The embryos displayed peripheral edema and pericardial effusion, consistent with heart failure (Fig. 1*B* and Fig. S3*A*). Overall cardiac patterning was preserved, but ventricular chamber size was severely reduced (Fig. 1*C* and Fig. S3*B*). In some cases, the hypoplasia affected both ventricles equally, whereas in other cases the left ventricle was more severely affected. Histological sectioning demonstrated a four-chambered heart with two atrioventricular and two outflow tract valves (Fig. 1*D* and Fig. S3*C*). The myocardium was markedly hypoplastic (Fig. 1*D*). Expression of MLC2A (MGI:MYL7) and MLC2V (MGI:MYL2), markers of atria and ventricular chamber specification, was unperturbed (Fig. S3*D*), indicating normal chamber specification. Rare survivors to E16.5 had ventricular septal defects (Fig. S3*C*).

To investigate the cellular mechanism underlying the myocardial hypoplasia, we performed immunostaining for markers of proliferation and apoptosis. Staining for histone H3 phosphorylated on serine 10 (pH3), a marker of M phase of the cell cycle, showed substantially reduced cardiomyocyte proliferation. Quantitative analysis showed that the fraction of cardiomyocytes positive for pH3 was reduced by twofold ($P < 0.05$; Fig. 1 *E* and *F*). This result was confirmed by analysis of cardiomyocyte uptake of 5-ethynyl-2'-deoxyuridine (EdU), a nucleotide analog that labels cells passing through S phase. EdU$^+$ cardiomyocytes were reduced by twofold in Yap1^{Tnnt2} mutant heart ($P < 0.05$; Fig. 1 *G* and *H*). Although YAP1 regulates apoptosis in other settings (14), apoptosis was not elevated in Yap1^{Tnnt2} cardiomyocytes, as assessed by TUNEL staining (Fig. S3*E*). Collectively, these data indicate that *Yap1* is essential for fetal cardiomyocyte proliferation.

To confirm that reduced cardiomyocyte proliferation led to decreased cardiomyocyte number, we used FACS to quantitate cardiomyocyte number in dissociated fetal hearts (Fig. S3 *F* and *G*). We took advantage of the Rosa26^{mTmG} (15) Cre reporter allele, in which Cre activity switches off baseline mRFP (membrane-bound RFP) and activates mGFP. GFP$^+$ cardiomyocyte number was reduced in Yap1$^{fl/fl}$::Tnnt2–Cre::Rosa26$^{mTmG/+}$ mutants compared with Yap1$^{fl/+}$::Tnnt2–Cre::Rosa26$^{mTmG/+}$ heterozygous controls. On the other hand, cardiomyocyte size, as measured by FACS forward scatter, did not differ between groups, suggesting that *Yap1* is not required for fetal cardiomyocyte size regulation (Fig. S3*H*). Collectively, these data indicate that *Yap1* is required to promote normal cardiomyocyte proliferation in the fetal heart.

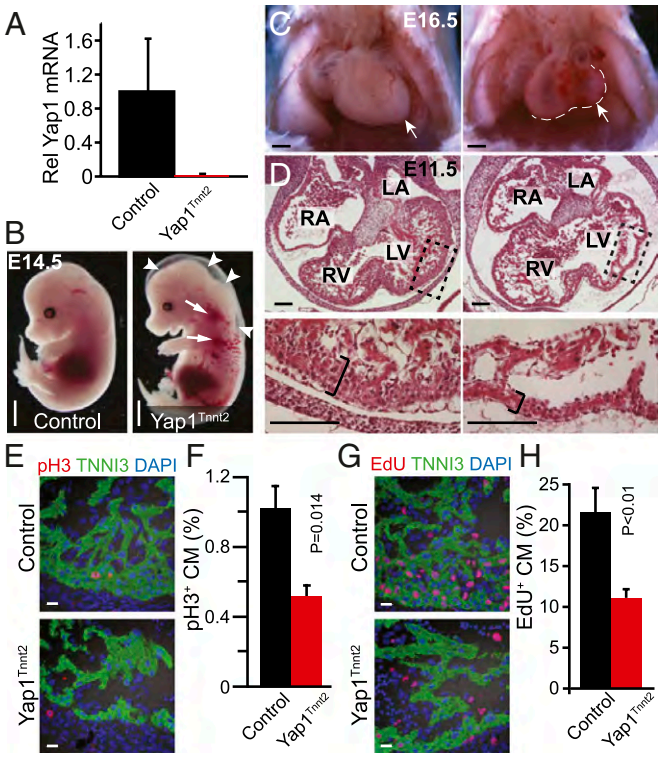

Fig. 1. Fetal cardiomyocyte-restricted inactivation of YAP1. (*A*) YAP1 mRNA levels in control (Yap1$^{fl/+}$::Tnnt2–Cre::Rosa26$^{mTmG/+}$) and Yap1^{Tnnt2} (Yap1$^{fl/fl}$::Tnnt2–Cre::Rosa26$^{mTmG/+}$) mutant cardiomyocytes, isolated at E12.5 by FACS for GFP. (*B*) *Hydrops fetalis* and peripheral hemorrhage in mutant embryo at E13.5. (Scale bar: 500 μm.) (*C*) Whole-mount image of E16.5 embryos after removal of the ventral chest wall, showing severe hypoplasia of mutant hearts. Hypoplasia was more severe in the left ventricle. For clarity, the white line indicates the edge of the heart. (Scale bar: 500 μm.) (*D*) Histological sections of E11.5 hearts. Magnifications show dramatic hypoplasia of compact myocardium in mutants (black brackets). (Scale bar: 100 μm.) (*E* and *F*) Reduced pH3 immunoreactivity in Yap1^{Tnnt2} mutant hearts. $n = 3$. (Scale bar in *E*: 10 μm.) (*G* and *H*) Reduced EdU staining in Yap1^{Tnnt2} mutant hearts. $n = 3$. (Scale bar: 10 μm.)

Cardiomyocyte Hypertrophic Growth Does Not Require *Yap1*. The fetal heart primarily grows through cardiomyocyte proliferation, but by postnatal day (P) 4, cardiomyocytes stop increasing in number and postnatal heart growth occurs by increased cardiomyocyte size (physiological hypertrophy) (16). Loss of *Yap1* in the fetal heart impaired cardiomyocyte proliferation but did not alter size. Therefore, we hypothesized that Yap1 is dispensable for physiological cardiomyocyte hypertrophy.

To test this hypothesis, we asked whether loss of *Yap1* has a cell-autonomous effect on postnatal cardiomyocyte growth. We sought to inactivate *Yap1* postnatally in a small fraction of cardiomyocytes, to avoid potentially triggering secondary effects that may result from widespread myocardial *Yap1* inactivation. A previous report indicated that retro-orbital administration of adenovirus to newborn pups leads to mosaic cardiomyocyte gene transfer (17). Retro-orbital delivery of Ad:Tnnt2–Cre to Yap1$^{fl/fl}$::Rosa26$^{mTmG/mTmG}$ pups activated the Cre-dependent mGFP reporter (and inactivated the baseline mRFP reporter) in a small fraction of cardiomyocytes, confirming that this technique is an effective means to achieve mosaic Cre-mediated recombination in the heart (Fig. 2*A*). We did not observe GFP expression outside of the heart.

To investigate the cell-autonomous role of *Yap1* in postnatal cardiomyocyte growth, we delivered Ad:Tnnt2–Cre to Yap1$^{fl/fl}$::Rosa26$^{mTmG/mTmG}$ pups at 3 d of life. At 4–6 wk of life, we compared the size of GFP$^+$ (*Yap1*-deficient) and GFP$^-$ (control) cardiomyocytes. In tissue sections, the cross-sectional areas of GFP$^+$ and GFP$^-$ cardiomyocytes did not differ significantly (Fig. 2*B*). Likewise, in dissociated cardiomyocyte preparations, cardiomyocyte projected area was not significantly altered (Fig. 2*C*). Thus, we conclude that *Yap1* is not required in a cell-autonomous manner for physiological cardiac hypertrophy.

Cardiomyocytes also increase in size in response to biomechanical stress, a process known as pathological hypertrophy. To determine whether YAP1 is essential for pathological hypertrophy, we performed ascending aortic constriction (AAC) on adult mice with mosaic inactivation of *Yap1*. The cross-sectional area of GFP$^-$ control cardiomyocytes was larger in AAC compared with unoperated hearts, consistent with activation of the hypertrophic response (Fig. S4). GFP$^+$ mutant cardiomyocytes were not significantly different in size compared with GFP$^-$ cardiomyocytes in either unoperated or AAC hearts (Fig. S4). Thus, YAP1 was dispensable for pathological cardiomyocyte hypertrophy.

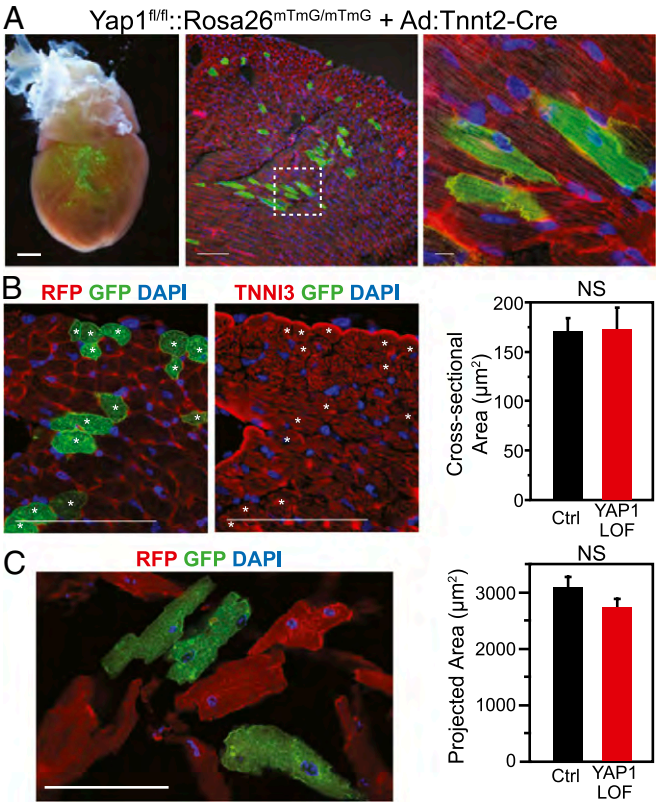

Fig. 2. YAP1 is not cell autonomously required for postnatal hypertrophic cardiomyocyte growth. (*A*) Mosaic Cre-mediated recombination after retro-orbital delivery of Ad:Tnnt2–Cre to P3 neonatal Yap1$^{fl/fl}$::Rosa26$^{mTmG/mTmG}$ mice. Boxed area is magnified in the *Right* panel. [Scale bars (from left): 500, 100, 10 μm.] (*B* and *C*) Hearts were examined at 4 wk (*B*) or 6 wk (*C*). Sizes of YAP1-deficient (mGFP^{+} and mRFP^{-}) and control (mGFP^{-} and mRFP^{+}) cardiomyocytes were measured in histological sections (cross-sectional area, *B*) or in dissociated preparations (projected area, *C*). No significant difference was detected between groups (NS; $n = 4$). (Scale bars: 100 μm.)

***Yap1* Gain of Function Stimulates Proliferation of Cultured Cardiomyocytes.** Given the essential role of *Yap1* in sustaining normal cardiomyocyte proliferation, we next asked whether increased *Yap1* is sufficient to drive cardiomyocyte proliferation. We developed an adenoviral vector to express activated YAP1 with an N-terminal triple FLAG epitope (Ad:FLAG–aYap1), where "activated YAP1" contained a serine 127 to alanine substitution that blocked Hippo pathway-mediated YAP1 inactivation (18). We confirmed expression in cultured neonatal rat cardiomyocytes (Fig. S5*A*). We used this tool to ask whether YAP1 gain of function enhances proliferation of fetal cardiomyocytes, which are normally actively proliferating. We transduced wild-type E16.5 fetal rat cardiomyocytes with Ad:FLAG–aYap1 or control (LacZ-expressing adenovirus, Ad:LacZ) adenovirus and measured the fraction of cells undergoing DNA synthesis [5′-bromodeoxyuridine (BrdU) uptake and mitosis (pH3 staining)]. Activated YAP1 significantly increased both measures of cardiomyocyte cell-cycle activity (Fig. S5*B*). Consistent with active fetal cardiomyocyte proliferation, the number of cardiomyocytes after 2 d of culture was greater in the activated YAP1 group compared with controls (Fig. S5*B*).

Having established that activated YAP1 was sufficient to stimulate proliferation of cardiomyocytes that were already actively engaged in the cell cycle, we next investigated whether *Yap1* gain of function could sustain proliferation in postnatal cardiomyocytes, which normally exit the cell cycle. Prior studies demonstrated that rat cardiomyocytes cease increasing in number by P4, although DNA synthesis and karyokinesis were detected above background levels for another 1–2 wk (16). Therefore, we prepared cardiomyocyte cultures from P4 neonatal rats. As expected, control neonatal rat ventricular cardiomyocytes treated with LacZ adenovirus rarely stained for pH3 or Aurora B, markers of M phase and cytokinesis, respectively, confirming that nearly all neonatal cardiomyocytes had exited the cell cycle (Fig. 3*A*). A small fraction (2%) of control neonatal cardiomyocytes were labeled with BrdU, consistent with low-level, persistent DNA synthesis activity (Fig. 3*A*). In contrast to control neonatal cardiomyocytes , aYAP1 neonatal cardiomyocytes exhibited far greater cell-cycle activity, with labeling indexes of 31%, 2.7%, and 2.1% for BrdU, pH3, and AuroraB, respectively (Fig. 3*A*). aYAP1 neonatal cardiomyocytes were observed at all stages of the cell cycle (Fig. S5*C*). Sequential imaging of the same fields over time demonstrated completion of cytokinesis in a subset of aYAP1-expressing but not control cardiomyocytes (Fig. 3*B* and Fig. S5*D*). We detected cell division events in 0/366 control cells, 0/267 LacZ cells, and 8/566 aYAP1 cells ($P = 0.015$; Fig. 3*B*). However, there was ongoing apoptosis in these cultures, which was reduced, but not eliminated, with FLAG–aYap1 expression (Fig. S5 *E* and *F*). As a result, there was an overall decline in cardiomyocyte number over the course of the experiment, although this decline was least in the aYAP1 group as a result of both increased proliferation and reduced apoptosis (Fig. S5*G*). Collectively, these data indicate that activated YAP1 continues to drive the cell cycle in postnatal cardiomyocytes, which normally have very little cell-cycle activity.

Activated Yap1 Stimulated Fetal Cardiomyocyte Proliferation in Vivo. Next, we asked whether YAP1 gain of function stimulates cardiomyocyte proliferation in intact fetal heart. We used a reported transgene (TetO–aYap1) (10) that expresses activated YAP1 (YAP1–[S127A]) from a doxycycline (Dox)-dependent promoter. In Tnnt2–Cre::Rosa26$^{fs\text{-}rtTA}$::TetO-aYap1 (abbreviated Yap1^{GOF}) mice, Dox induced expression of activated YAP1 selectively in cardiomyocytes (Fig. S6 *A* and *B*). Administration of Dox to YAP1^{GOF} fetuses starting at E8.5 resulted in fetal demise by E15.5. At E12.5, mutant fetuses exhibited peripheral hemorrhage, hepatic congestion, and cardiomegaly (Fig. S6*C*). Histological examination revealed dramatic myocardial overgrowth with moderate thickening of the compact myocardium and marked expansion of the trabecular myocardium, causing near chamber obliteration (Fig. 4*A*, arrowheads).

To evaluate the cellular mechanism for myocardial expansion, we performed immunostaining for proliferation markers. Cardiomyocyte proliferation was significantly increased in Yap1^{GOF}

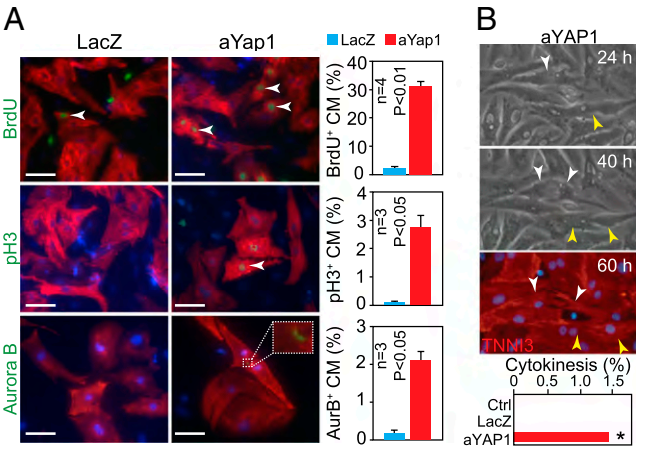

Fig. 3. Activated YAP1 stimulated proliferation of cultured cardiomyocytes. (*A*) aYAP1 stimulated cell-cycle activity of P4 neonatal rat cardiomyocytes, as measured by immunostaining for BrdU, pH3, and Aurora B (AurB). (Scale bars: 50 μm.) (*B*) Sequential imaging of cardiomyocytes at the indicated times after aYAP1 transduction. Two cardiomyocytes underwent cytokinesis in this field (arrowheads). At 60 h, cultures were immunostained for the cardiomyocyte marker TNNI3. Full imaging series is in Fig. S5*D*. *$P = 0.015$.

fetal heart and control mice exposed to Dox starting at E8.5, as measured by the M-phase marker pH3 (Fig. 4 *C* and *D*). The S-phase marker EdU also increased by twofold, which approached, but did not achieve, statistical significance ($P = 0.07$, $n = 3$; Fig. 4 *B* and *D*). Thus, *Yap1* gain of function in fetal heart was sufficient to enhance cardiomyocyte proliferation, causing marked cardiomyocyte hyperplasia.

In control heart, the proliferation rate of cardiomyocytes was substantially lower in trabecular compared with compact myocardium (Fig. 4 *B* and *C*), indicating that, even in fetal heart, cardiomyocytes in specific compartments are exiting the cell cycle. This finding was particularly evident by pH3 staining, where it was very rare to observe $pH3^+$ trabecular cardiomyocytes. In contrast, proliferating mutant trabecular cardiomyocytes were readily observed in $Yap1^{GOF}$ (Fig. 4 *B* and *C*). Quantitation of EdU uptake showed that $Yap1^{GOF}$ stimulated trabecular myocardial proliferation fivefold, to levels comparable with compact myocardium (Fig. 4*E*). These data indicate that *Yap1* gain of function is sufficient to sustain cell-cycle activity in trabecular cardiomyocytes, causing tremendous expansion of trabecular myocardium.

Cardiomyocyte proliferation is often linked to differentiation. Trabecular and compact cardiomyocytes have distinct gene expression programs. One marker expressed in trabecular, but not compact, myocardium is *Nppa*. In situ hybridization showed that *Nppa* expression was strongly down-regulated in trabecular myocardium expressing activated YAP1 (Fig. S6*D*), consistent with impaired activation of the trabecular myocardial gene program.

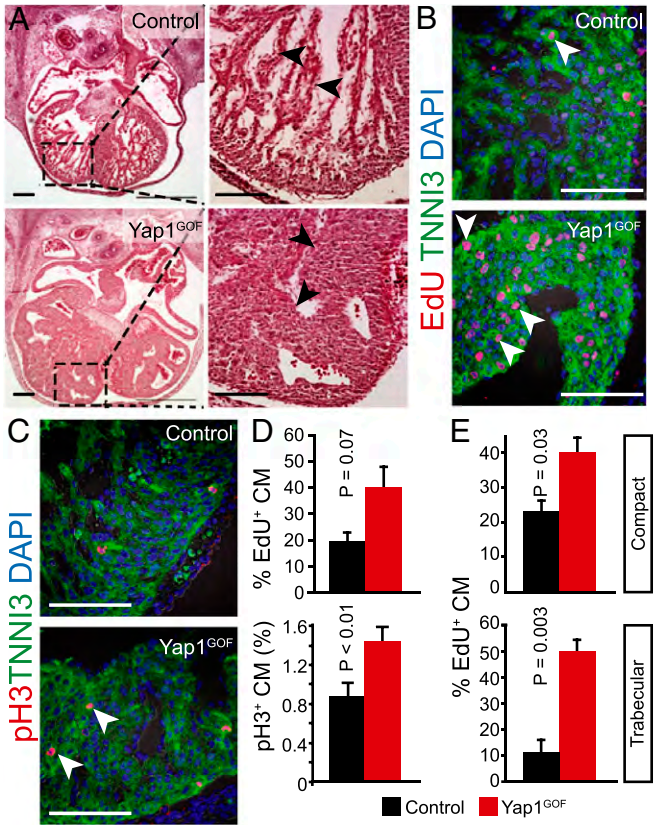

Fig. 4. Fetal YAP1 gain of function stimulated cardiomyocyte proliferation in vivo. (*A*) Histological sections of $Yap1^{GOF}$ heart at E12.5 revealed marked hypertrabeculation that nearly obliterated the cardiac chambers. Black arrowheads indicate trabecular myocardium. (*B* and *C*) pH3 and EdU staining showed elevated cardiomyocyte proliferation, particularly in trabecular myocardium (white arrowheads). (*D*) Quantitation of *B* and *C* for ventricular myocardium (compact and trabecular pooled). (*E*) Quantitation of cardiomyocyte EdU uptake in compact vs. trabecular myocardium. (Scale bars: 100 μm.)

Activated YAP1 Stimulated Postnatal Cardiomyocyte Proliferation in Vivo. In both P4 neonatal cardiomyocytes and fetal trabecular cardiomyocytes, YAP1 gain of function was sufficient to drive proliferation of cardiomyocytes that normally have exited the cell cycle. Therefore, we asked whether YAP1 gain of function was sufficient to drive proliferation of cardiomyocytes in the intact infant heart, in which cardiomyocytes are normally exiting from the cell cycle. We treated $Yap1^{GOF}$ mice with Dox from P5 and analyzed hearts at P15. Heart weight was increased in $YAP1^{GOF}$ mice compared with the controls (heart weight/body weight, 9.0 ± 0.1 vs. 7.5 ± 0.5 mg/g; $P = 0.03$; $n = 3$). This result was not due to cardiomyocyte hypertrophy, because cardiomyocyte size did not differ significantly between $Yap1^{GOF}$ and control groups (Fig. 5*A*). EdU labeling index was increased nearly twofold in $YAP1^{GOF}$ cardiomyocytes, indicative of a higher fraction of cardiomyocytes passing through S phase during the 24-h EdU pulse (Fig. 5*B*). This finding was corroborated by staining for the M-phase marker pH3, which showed 15-fold increased cell-cycle activity in $Yap1^{GOF}$ cardiomyocytes (Fig. 5*C*). Collectively, these data indicate that YAP1 gain of function increased postnatal cardiomyocyte proliferation.

YAP1 Regulates Expression of Cell-Cycle Genes in Cardiomyocytes. To begin to identify genes downstream of *Yap1* that stimulate cardiomyocyte proliferation, we performed microarray expression profiling in *Yap1* gain of function. We used the Affymetrix Rat Gene 1.0 ST microarray to compare gene expression between P4 neonatal cardiomyocyte transduced with FLAG-aYap1 or LacZ (control). Gene Set Enrichment Analysis (19) of the expression profiles using gene sets for manually curated canonical pathways indicated that the large majority of the sets significantly enriched by activated YAP1 related to cell proliferation and DNA synthesis (Fig. S7 *A* and *B*). Hierarchical clustering using cell cycle-related genes clustered samples into control or aYAP1 groups (Fig. 6*A* and Fig. S7*C*). Comparison of individual gene expression levels between groups identified 1,263 differentially expressed probe sets corresponding to 1,057 known genes ($n = 4$; $P < 0.005$ and fold change >50%; Dataset S1). We validated differential expression of a subset of cell cycle-regulated genes by qRT-PCR. Of eight genes tested that were differentially expressed by

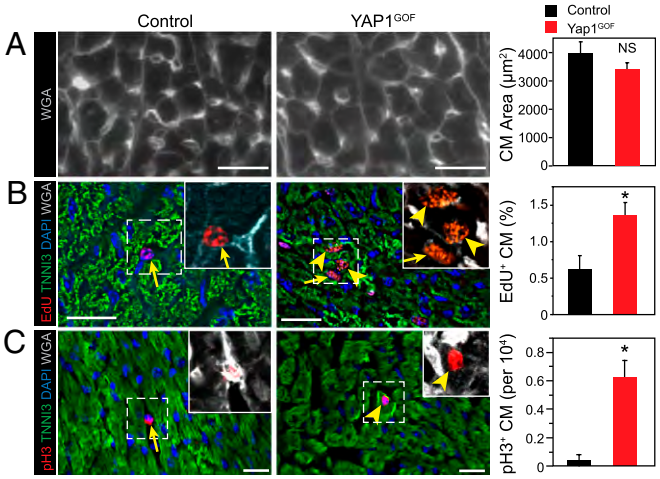

Fig. 5. Postnatal YAP1 gain of function stimulated cardiomyocyte proliferation in vivo. $YAP1^{GOF}$ or control mice were treated with Dox from P5 to P15, when they were analyzed for cardiomyocyte proliferation. (*A*) The size of YAP1 gain-of-function cardiomyocytes was indistinguishable from control. Wheat germ agglutinin (WGA) staining outlined the edges of cardiomyocytes. (*B* and *C*) Cardiomyocyte proliferation was increased based on EdU labeling index (*B*, deconvolution) and pH3 staining (*C*, confocal). Arrowheads indicate cardiomyocytes and arrows nonmyocytes. *Insets* show magnifications of boxed regions, with cardiomyocyte borders highlighted by WGA staining. $n = 3$. $^*P < 0.05$. (Scale bars: 20 μm.)

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microarray, six were validated by qRT-PCR (Fig. 6*B*). Among upregulated cell-cycle genes were Cyclin A2 (*CcnA2*), Cyclin B1 (*CcnB1*), and Cyclin-dependent kinase 1 (*Cdc2*), which have been shown to be sufficient to drive limited cardiomyocyte proliferation in postnatal hearts (20–22). Cyclins D1 and D2, which also promote cardiomyocyte proliferation (23, 24), approached but did not reach our cutoff thresholds for differential expression by microarray ($P = 0.003$–0.004 and fold change 1.4–1.5). These data indicate that YAP1 stimulates cardiomyocyte proliferation through the coordinate activation of a number of cell-cycle genes.

YAP1 Stimulation of Cardiomyocyte Proliferation Requires TEAD Interaction. In many contexts, coactivator YAP1 binds to TEAD family factors to stimulate cell proliferation (2). However, in some cases YAP1 regulation of cell growth is independent of TEAD factors (8). To assess the role of YAP1–TEAD interactions in promoting cardiomyocyte proliferation, we expressed GFP fused to a region of YAP1 sufficient to bind TEAD (25). We reasoned that this peptide, corresponding to amino acid residues 47–155 of YAP1, would interfere with YAP1–TEAD interaction (Fig. 7*A*) and named the peptide YTIP (YAP1–TEAD Interfering Peptide). In coimmunoprecipitation experiments, we confirmed that GFP–YTIP impaired YAP1–TEAD1 interaction (Fig. 7*B*).

We next measured the effect of GFP–YTIP on activated YAP1 stimulation of cardiomyocyte proliferation. GFP–YTIP strongly attenuated the stimulatory effect of activated YAP1 on proliferation of P4 neonatal cardiomyocytes, as measured by BrdU uptake and pH3 immunoreactivity (Fig. 7*C*). The antiproliferative effect of GFP–YTIP was paralleled by decreased activation of cell-cycle genes *Cdk1* and *Cyclin A2* by aYAP1 (Fig. 7*D*). These data indicate that YAP1 mitogenic activity in cardiomyocytes requires YAP1–TEAD interaction.

Substitution of YAP1 serine 79 by alanine selectively abolished TEAD interaction, and a $Yap1^{S79A}$ allele was unable to restore proliferation defects caused by ablation of wild-type Yap1 (11). We generated $Yap1^{fl/S79A}$::Tnnt2–Cre embryos, in which only the mutant, and not the wild-type, YAP1 protein would be expressed in the heart. These mutant mice exhibited cardiomyocyte hypoplasia at least comparable in severity to that caused by cardiomyocyte Yap1 ablation (Fig. 7*E*). These data indicate that YAP1 stimulation of cardiomyocyte proliferation requires TEAD interaction, both in vitro and in vivo.

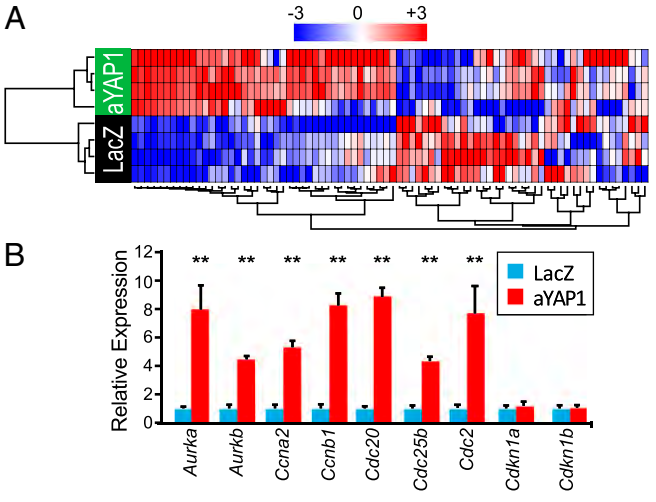

Fig. 6. YAP1 promotes expression of cell-cycle genes through interaction with TEAD1. (*A*) Heat map displaying two-way hierarchical clustering of cell-cycle genes in P4 neonatal cardiomyocytes expressing either aYAP1 or LacZ (control). Expression of cell-cycle genes segregated samples into treatment groups. (*B*) Validation of differential expression of cell-cycle genes by qRT-PCR. $^{**}P < 0.001$. $n = 4$.

Discussion

Growth of the mammalian heart occurs through both cardiomyocyte proliferation and hypertrophy, with proliferation and hypertrophy primarily driving fetal and postnatal heart growth, respectively. YAP1 has been shown to promote cellular proliferation and organ growth in multiple systems in flies and mammals (2). Although YAP1 regulation of organ growth through control of cellular proliferation has been studied in depth, little has been reported about YAP1 regulation of hypertrophic organ growth. Our results indicate that YAP1 is both necessary and sufficient for fetal cardiomyocyte proliferation. However, YAP1 is neither necessary nor sufficient for postnatal hypertrophic growth of the heart. Thus, our data indicate that YAP1 regulates organ size predominantly by controlling cell number.

Normal heart development requires precisely regulated regional heart growth. Selective hypoplasia of heart structures are seen in congenital heart disease such as hypoplastic left heart syndrome. Our study showed that proper regulation of YAP1 activity is critical for normal growth of the fetal heart. Interestingly, overall size of the left ventricular chamber was more severely affected in some $YAP1^{TNT}$ loss-of-function mutants (e.g., Fig. 1*C* and Fig. S3*D*). Moreover, it is likely that YAP1 activity is regionally controlled, so that localized disruption of YAP1 regulation could lead to selective chamber hypoplasia. Thus, mutation of genes in the YAP pathway may participate in pathogenesis of congenital heart disease involving abnormalities of myocardial growth.

Myocardial trabeculation is essential for fetal heart growth and function. Cardiomyocytes in trabeculae exit the cell cycle through unknown mechanisms, suggesting that growth of trabeculae occurs from addition of cardiomyocytes from compact myocardium. Neuregulin signaling to ErbB2/ErbB4 is essential for myocardial trabeculation (26, 27), and recent studies suggest that ErbB2 acts by promoting compact myocardial proliferation and directional cardiomyocyte migration into trabeculae (28). Our data indicate that

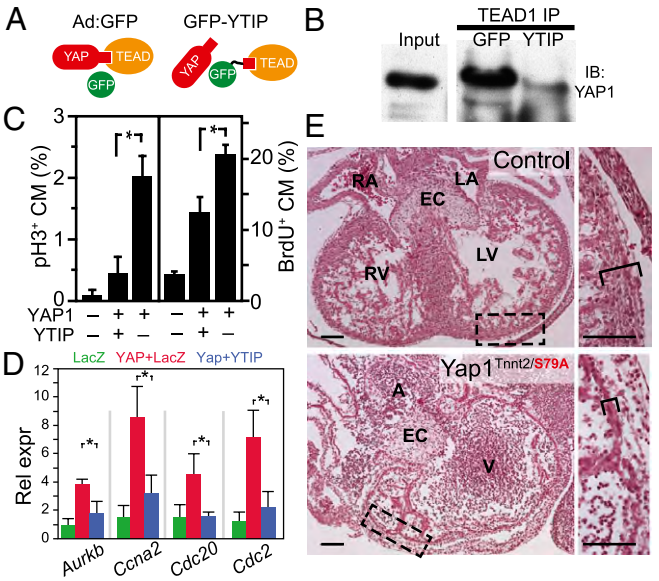

Fig. 7. YAP1 stimulation of cardiomyocyte proliferation requires TEAD interaction. (*A*) YAP1-TEAD inhibitory peptide (YTIP) strategy. (*B*) Endogenous TEAD1 and YAP1 interaction was blocked by Ad:GFP–YTIP in MES13 cells. (*C*) aYAP1 stimulation of P4 neonatal cardiomyocyte proliferation was attenuated by GFP–YTIP, as measured by BrdU and pH3 immunostaining. $^{*}P < 0.01$. $n = 3$. (*D*) Inhibition of TEAD interaction reduced expression of YAP1-activated cell-cycle genes, as measured by qRT-PCR. $^{*}P < 0.05$. $n = 3$. (*E*) Cardiomyocyte $YAP1^{S79A}$, deficient in TEAD interaction, did not support normal fetal myocardial growth. $Yap1^{Tnnt2/S79A}$ indicates the genotype $Yap1^{fl/S79A}$::Tnnt2–Cre. H&E-stained sections of E12.5 heart are shown. (*Right*) Boxed areas are enlarged. Brackets indicate compact myocardial thickness. (Scale bars: 100 μm.)

YAP1 is essential for regulating myocardial trabeculation, because trabeculation was reduced in $YAP1^{TNT}$ mutants. This reduction of trabeculation may be a consequence of reduced proliferation of cardiomyocytes in compact myocardium. YAP1 activation in cardiomyocytes prevented trabecular cardiomyocyte cell-cycle exit, suggesting that negative regulation of the YAP1 pathway is essential for limiting trabecular myocardial expansion. Interestingly, YAP1 activation also prevented expression of the trabecular cardiomyocyte marker *Nppa*, suggesting that YAP1-induced trabecular proliferation is linked to impaired differentiation. Abnormalities of YAP1 regulation may contribute to myocardial noncompaction, which is seen in some forms of human cardiomyopathy.

The Hippo pathway is the best-known regulator of YAP1 activity. However, these kinases have multiple downstream targets and influence diverse processes such as apoptosis and autophagy (29). Indeed, the roles of Hippo pathway kinases LATS and MST in regulating these later processes have been explored by cardiomyocyte-restricted transgenic gain and inhibition of function (30, 31). Recently, cardiac-restricted inactivation of the Hippo pathway components *Sav1*, *Lats2*, or *Mst1/2* was shown to cause cardiac overgrowth (32). Thus, the findings of that study combined with those of the present study indicate that Hippo kinases restrain the proliferative activity of YAP1 on fetal cardiomyocytes.

Gene expression profiling showed that activated YAP1 potently and coordinately activated gene programs that promote cell-cycle activity. YAP1 activates transcription by binding to DNA-binding transcription factors, with TEAD1-4, orthologs of *Drosophila Scalloped*, being the best known (2). However, not all proliferative activities of YAP1 or its *Drosophila* ortholog Yorkie are mediated by TEAD/Scalloped. For example, *Yorkie* but not *Scalloped*, is required for growth of *Drosophila* imaginal discs (8). Using a dominant negative peptide that blocks YAP1–TEAD and a YAP1 point mutation-defective TEAD interaction, we showed that YAP1 stimulation of cardiomyocyte proliferation requires its interaction with TEAD.

Activated YAP1 potently promoted proliferation of fetal trabecular and neonatal ventricular cardiomyocytes that normally exit the cell cycle. These data show that activation of YAP1 can overcome signals that normally lead cardiomyocytes to exit the cell cycle. However, a limitation of the current study is that we cannot distinguish whether YAP1 is preventing cell-cycle withdrawal or reinitiating cell-cycle reentry. This limitation will need to be addressed further to test the hypothesis that activated YAP1 can induce cell-cycle reentry in adult, fully mature cardiomyocytes, with important potential ramifications for regenerative approaches to heart injury.

Materials and Methods

Please see *SI Materials and Methods* for details.

All animal procedures were approved by the Children's Hospital Boston Institutional Animal Care and Use Committee. TetO-YAP1 (10), $Yap1^{flox}$ (11), $Yap1^{S79A}$ (11), $Rosa26^{mTmG}$ (15), $Rosa26^{fs\text{-}rtTA}$ (33), and Tnnt2-Cre (13) alleles were described.

Tnnt2–Cre adenovirus was constructed using the rat Tnnt2 promoter (34) and the AdEasy system (Stratagene). 3xFlag–YAP1 adenovirus expressed S127A-mutated human YAP1 cDNA with an N-terminal triple FLAG epitope tag. The GFP–YTIP construct contained eGFP fused to residues 47–155 of human YAP1. Cesium chloride gradient-purified viruses were titered with the AdEasy titer kit (Stratagene). Retro-orbital adenoviral injection to neonatal pups was performed as described (17).

Fetal and neonatal rat cardiomyocyte culture was performed by using the Neomyts cardiomyocyte dissociation kit (Cellutron). Adult cardiomyocytes were isolated by antegrade collagenase perfusion and purified by differential centrifugation.

Antibody sources are listed in Table S1. Primers for quantitative RT-PCR are in Table S2. Total RNA was isolated by using the RNeasy kit (Qiagen) and hybridized to Affymetrix microarrays (Rat Gene 1.0 ST).

Results are expressed as mean ± SEM. Two group comparisons were performed using Student *t* test.

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