THE UNIVERSITY OF CHICAGO

BAP1 REGULATES THE HAIR CYCLE THROUGH THE TGFβ SIGNALING PATHWAY

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Table of Contents

List of Figures

Chapter 1: Introduction

1.1 The Structure and Development of the Skin

The skin, the body's largest organ, serves a variety of functions, including non-specific immunity, protection from dehydration, and general protection of an organism from its environment. Physiologically, it is a multi-layered structure consisting of subcomponents called the epidermis, dermis, and hypodermis (apical to basal), each possessing unique cell types and proteinous ultrastructure(Lopez-Ojeda et al. 2022). Embryologically the skin is derived principally from the ectoderm layer of the gastrula with some components of the epidermis, dermis and hypodermis being contributed by the somites and neural crest cells(Mort, Jackson, and Patton 2015; Yousef, Alhajj, and Sharma 2022).

In the case of chordates, soon after gastrulation ectodermal cells that receive Wingless integrated (Wnt) signaling, which blocks the response to fibroblast growth factor (FGF), express bone morphogenic proteins (BMPs) and are thus fated to develop as epidermal cells; other ectodermal cells that fail to receive Wnt signals are specified as neural tissues(Elaine Fuchs 2007). As a result of the afore described process, the immature ectoderm consists of a single layer of cuboidal epithelial cells that progress through a series of stratification and differentiation events that form the mature epidermis(Richardson et al. 2014). The mature epidermis, the outermost layer of the skin, forms a waterproof barrier containing keratinocytes, melanocytes, and Langerhans cells. The dermis, located beneath the epidermis, is vascularized and contains connective tissue, fibroblasts, skin appendages and lymphatic vessels (Fig 1). The deepest layer of the skin, the hypodermis, is a fatty layer that contains a large amount of connective tissue Each the dermal and

epidermal layers are, in turn, comprised of multiple layers; the dermis contains two subcomponent layers while, the epidermis has four to five depending on where it is located on the body(Lopez-Ojeda et al. 2022). The layers of the epidermis include the basal, spinous, granular, and stratum corneum (Lopez-Ojeda et al. 2022; Yousef, Alhajj, and Sharma 2022) (Fig. 1); only the basal layer of the skin contains proliferative cells known as keratinocytes which travel to the apical-most layer over the course of about four weeks and are then shed(Elaine Fuchs 2016). These keratinocytes have been found to be readily isolatable and can be cultured in vitro for hundreds of passages without losing their stem-ness(Elaine Fuchs 2016).

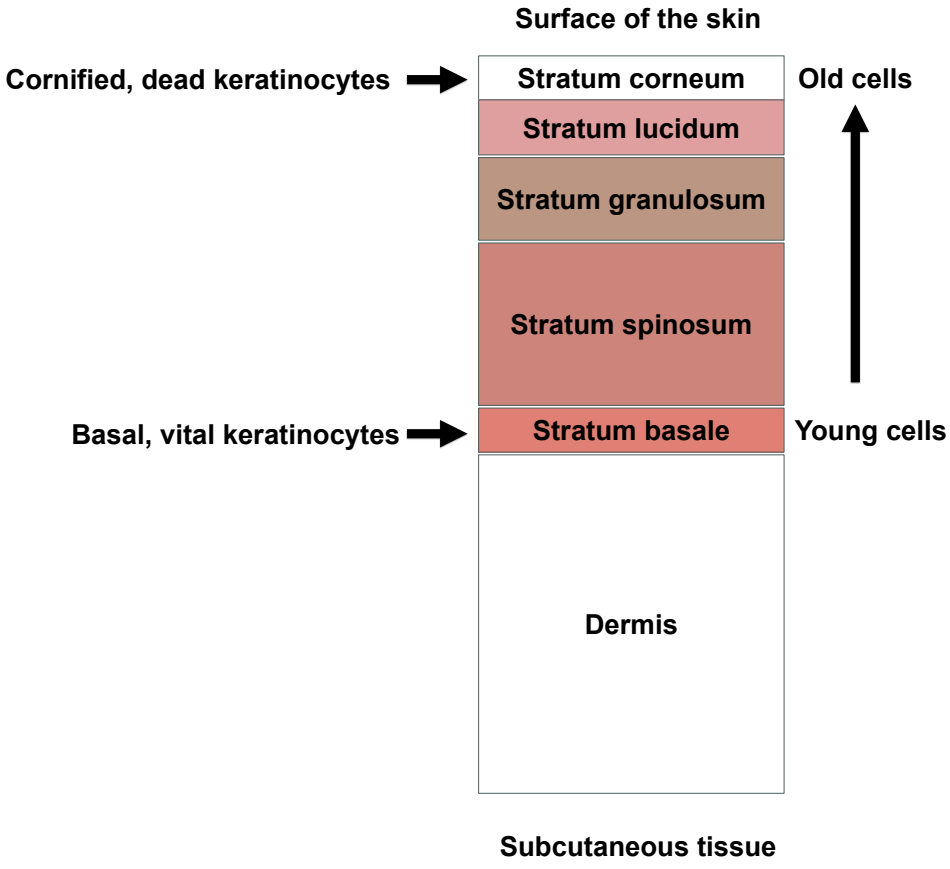

Figure 1 Structure of Mammalian Skin. A schematic showing the apical to basal distribution of the skin epidermis layers relative to one another and the dermis.

1.2 The Hair Follicle

1.2.1 The Structure and Development of The Hair Follicle

As notable as its component layers are the two major groups of appendages the skin contains, these being two subsets of sweat glands (apocrine glands and eccrine glands) and the hair follicles; each of which are, in mouse, derived from distinct pools of equipotent placodes within the epidermis of the embryonic mouse(Lu et al. 2016). The apocrine glands are, for the most part, not active until the onset of puberty, unlike the eccrine glands, which become active shortly after birth and serve a thermoregulatory function(Hodge, Sanvictores, and Brodell 2022); the latter is considered essential(Cui and Schlessinger 2015).

Despite originating in the epidermis, at maturity hair follicles producing terminal hairs are located within the skin dermis. These follicles consist of three major segments, these being: the infundibulum, the isthmus, and the inferior segment which includes the hair bulb(Schneider, Schmidt-Ullrich, and Paus 2009). In addition to possessing distinct regions along the length of the follicle, the hair shaft is surrounded by concentric layers of cells of differing identities possessing unique secretomes which contribute to the life cycle of the hair follicle, these cells are majorly contained within the outer root sheath (ORS), companion layer (CL), and inner root sheath (INS) (Mahjour, Ghaffarpasand, and Wang 2012; Jiao 2019)(Fig. 2).

1.2.2 Regulation of The Hair Cycle

Given the importance and complexity of the skin, its appendages, and their development, it has been the subject of numerous research studies of antiquity. It continues to be an intensely investigated system today(Morrow and Lechler 2015). As a result, many signaling pathways such as sonic hedgehog (SHH), Wnt, and those associated with transforming growth factor β (TGFβ)

are conclusively known to have extensive involvement in the development and maintenance of the various infrastructural features of the hair follicle mini organ(Veltri, Lang, and Lien 2018; Lu et al. 2016).

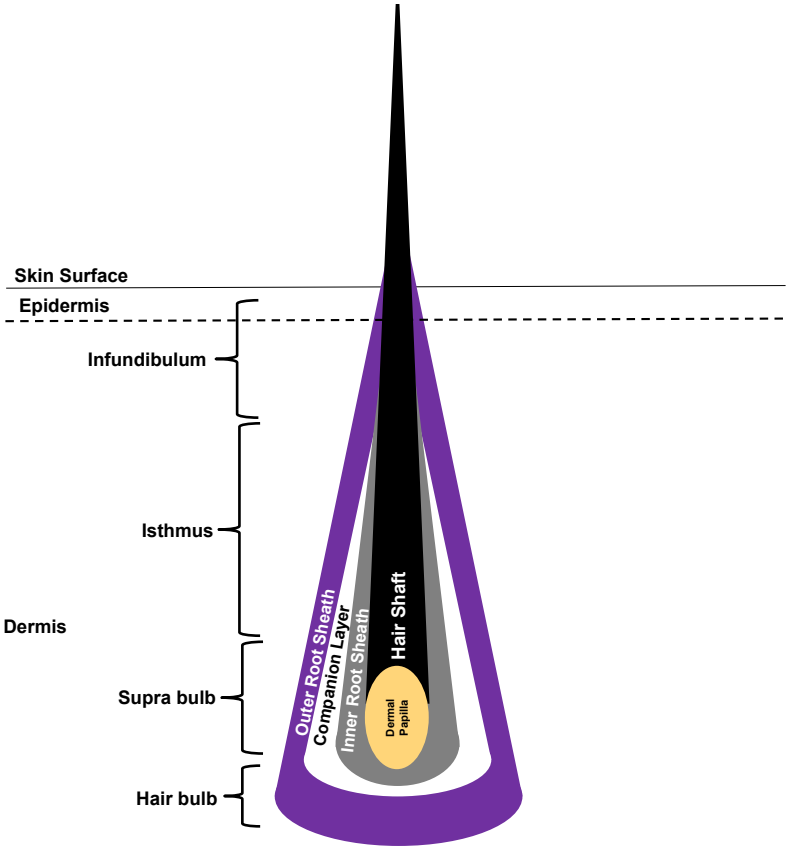

Figure 2. **Structure of the Hair Follicle**. A schematic showing the anatomy of an anagen-stage hair follicle as well as the segments of the hair shaft relative to the skin's surface.

The hair follicle is a dynamic mini organ which cycles through three phases known as anagen (active growth), catagen (regression), and telogen (stasis) with a fourth phase known as exogen (shedding) only occasionally occurring (Fig, 3)(Botchkareva, Ahluwalia, and Shander 2006); of note is the fact that these changes take place according to a very precise time scale(Müller-Röver et al. 2001).

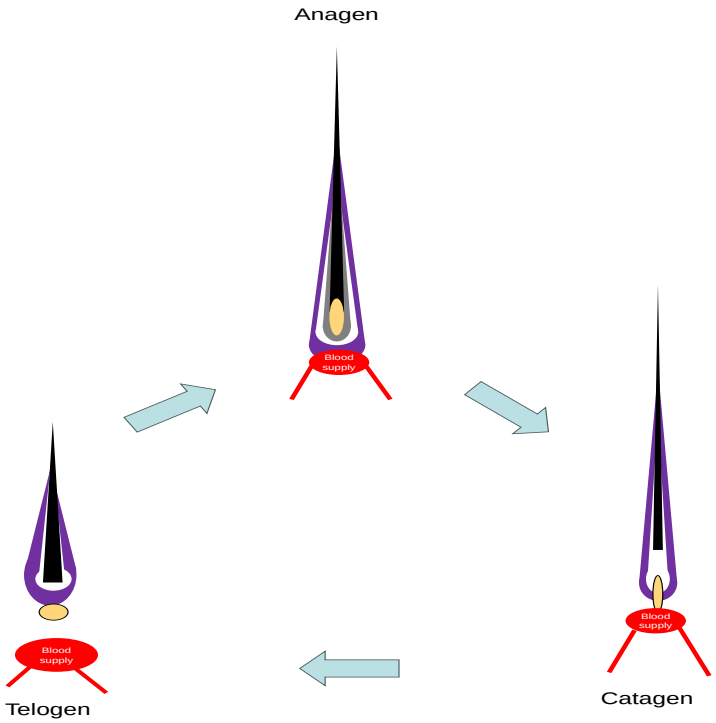

Figure 3. **The Hair Follicle Cycle**. A diagram showing the cyclic progression of the hair follicle cycle as well as the approximate changes to the major cell layers and hair follicle morphology.

The hair follicle cycle in mouse begins first with morphogenesis which occurs beginning in embryonic development and is completed by postnatal day 10(Müller-Röver et al. 2001; Rishikaysh et al. 2014). Herein, three steps occurring over eight distinct stages occurs within the embryonic skin(Müller-Röver et al. 2001). First, induction occurs when Wnt signals arise within the mesenchymal cells directing the thickening of epithelial cells into a skin placode while dermal cells gather into a dermal condensate just below(Xiangyu Lin, Zhu, and He 2022; Park 2022; Rishikaysh et al. 2014). Next, organogenesis occurs when a complex interplay of signals are exchanged between the dermal condensate and placode including both Wnt and SHH causing each to grow downward while surrounding inhibitory signals such as BMP an Dickkopf (Dkk) restrict this type of development to cause a typical array-like pattern of hair follicles in the embryonic

skin(Park 2022). Finally, after birth, in cytodifferentiation the associated dermal condensate is enveloped in follicular epithelial cells forming a dermal papilla (DP) and in turn instructs the structures in the overlying ectoderm to shape and develop the hair follicle such that it develops the diverse cell populations contained within the concentric layers of the mature hair follicle(Park 2022; Müller-Röver et al. 2001). At maturity, these transcriptionally distinct cell populations contained principally within the ORS, CP, and IRS, respectively(Xiangyu Lin, Zhu, and He 2022), contribute uniquely to the persistence and growth of the hair follicle over the term of morphogenesis(Veijouye et al. 2017).

At around 16 days post birth the first catagen begins starting from the head of the mouse and continuing in a wavefront-like fashion along the body of the animal to the tail (Plikus and Chuong 2008). Herein, the populations of cells within the hair follicle begin to fluctuate due to the keratinocytes of the ORS secreting several catagen-inducing factors before and during this phase including FGF5 short isoform, IGF Binding Protein 3 (IGFBP3), TGFβ1/2 and others. The primary targets of these signals are the keratinocytes contained within the IRS which become strongly TUNEL+ and express factors implicated in hair follicle keratinocyte apoptosis; one major impact of this the shortening of the IRS followed by the clearance of the apoptotic cells(Rishikaysh et al. 2014; Müller-Röver et al. 2001). Transcriptional and secretomic changes that occur during this time led to several morphological changes resulting in the club hairs observed in late catagen. Of note is the stem cell population located between the opening of the sebaceous gland and attachment site of the arrector pili muscle known as the bulge niche which is marked by Cluster of differentiation (CD) 34, SRY-Box transcription Factor 9 (SOX9), Leucine-rich repeat-containing G-protein-coupled Receptor 5 (LGR5), and cytokeratins 15 and 19; this population is considered

permanent and persists through catagen to ultimately multipotently contribute to tissue regeneration(Veijouye et al. 2017).

By post-natal day 19 evidence of hair follicles moving into the telogen phase begins to appear starting in the at the snout end of the animal. During the telogen the hair follicles are very short with a compact ball-shaped DP and do not display any IRS tissue(Müller-Röver et al. 2001). During this time the DP and surrounding fibroblasts supply BMP to sustains a Wnt-low environment which maintains hair follicle cell quiescence(P. Wu et al. 2019; Quist and Quist 2021). The first telogen lasts until around post-natal day 28(Müller-Röver et al. 2001) when Wnt activation breaks the hair follicle cells out of stasis and stimulates the transition from telogen to the first genuine anagen where SHH signal induction becomes prominent(Oro and Higgins 2003; P. Wu et al. 2019).

Unfortunately, much remains unknown about many aspects of the molecular signals which regulate each stage of the hair follicle cycle.

1.2.3 Transforming Growth Factor β Signaling Pathway

The TGFβ signaling pathway holds a large number of roles in organismal development and homeostasis(Niimori et al. 2012; Clayton et al. 2020; Foitzik et al. 2000) .Canonical TGFβ signaling involves one of three ligand isoforms (TGFβ1/2/3), their paired receptors (TGFβr type 1 and 2), and their secondary messengers (SMAD2/3)(Aykul and Martinez-Hackert 2016). Herein, a TGFβ ligand engages the extracellular domain of one of the 5 types of TGFβr2(Aykul and Martinez-Hackert 2016; Munir et al. 2004), a constitutively active serine/threonine kinase(Vander Ark, Cao, and Li 2018), causing it to activate one of the 7 types of TGFβr1(Aykul and Martinez-Hackert 2016) via phosphorylation resulting in the receptors and ligand forming an activated

complex(Di Guglielmo et al. 2003; Vander Ark, Cao, and Li 2018; Wrana et al. 1992). Together the activated TGFβ receptors form a heterotetrametric complex (comprised of two TGFβr1/2 heterodimers)(Huang et al. 2011) whose resultant conformational changes to the paired cytoplasmic domains of the type 1 and 2 TGFβ receptors allow the docking and phosphorylation of receptor SMADs 2 and 3 recruited by the SMAD-Anchor for Receptor Activation (SARA)(Aykul and Martinez-Hackert 2016). Receptor SMADs activated by TGFβ signaling form complexes with co-SMAD4 which then translocates into the nucleus where it influences gene expression; SMAD-independent, non-canonical TGFβ signaling using ERK1/2, mTOR, JNK, ROCK, and p38 as downstream effectors have also been documented(Y. E. Zhang 2009).

The negative regulation of the TGFβ pathway is accomplished in two ways: (1) activated TGFβ receptor heterotetramers are endocytosed in clathrin coated vesicles to limit continued receptor stimulation then either recycled or degraded (M. Y. Wu and Hill 2009) ;(2)Inhibitory SMADs (I-SMADS), including SMAD6 but particularly SMAD7, contribute to TGFβ signaling by negatively regulating the formation and accumulation of activated SMADs and TGFβr1/2 complexes(Kavsak et al. 2000; Di Guglielmo et al. 2003; Ying Zhang et al. 2001). I-SMADs prevent TGFβ signaling by preventing new R-SMAD phosphorylation events through blocking the TGFβ receptor activated complex cytoplasmic region in addition to preventing activated SMAD2/3 signaling by preventing it from complexing with co-SMAD4(Thakur et al. 2020; Kavsak et al. 2000). Regarding the facilitation of pathway component degradation, I-SMADs mediate the ubiquitination of TGFβ receptor complexes and activated R-SMADs by recruiting ubiquitin ligases SMAD Ubiquitin Regulatory Factor (SMURF) 1 and 2 as well as NEDD4L(Kavsak et al. 2000; Ying Zhang et al. 2001; Gao et al. 2009; Kuratomi et al. 2005) .

In the context of proteins contained within the cytosol, ubiquitination may create several signals including those pertaining to cell cycle regulation, signal transduction, protein-trafficking and most notably protein degradation(Ying Zhang et al. 2001; Ronai 2016). Generally, mono-ubiquitin groups are linked to protein sorting and trafficking while poly- ubiquitin chains are associated with targeting for proteasomal degradation; the proportion of mono-ubiquitinated to poly-ubiquitinated proteins degraded by proteasomes in human cells is 50:50 while in yeast this figure is 25:75(Ronai 2016). Relating specifically to the regulation of TGFβ signaling, pathway component turnover is both a well-documented and well-studied phenomenon. SMURFs 1 and 2 were first identified in 1999 and 2000, respectively, and are two HECT class ubiquitin ligases which contribute to ubiquitin-mediated SMAD regulation(Kavsak et al. 2000; Xia Lin, Liang, and Feng 2000; Zhu et al. 1999). SMURF1 is most associated with the degradation of BMP-activated SMADs while SMURF2 is considered a minor contributor to the degradation of TGFβ-activated SMADs with the major contributor being HECT E3 ubiquitin ligase NEDD4L(Gao et al. 2009; Kuratomi et al. 2005; Xia Lin, Liang, and Feng 2000) ;NEDD4L specifically targets SMAD proteins for ubiquitination based on their phosphorylated conformation(Gao et al. 2009). These ubiquitin ligases polyubiquitinate activated SMADs both within the nucleus or within the cytoplasm as well as activated receptor complexes to dictate their turnover(Inman and Hill 2002; Hu et al. 2021).

The nature of TGFβ, its signaling, and its regulation have been of interest since 1981 when it was first identified for its notable effects on cancer cells, wound healing, and immunity(Roberts 2023). In mouse, the exact roles of TGFβ family proteins have been under intense investigation which utilized transgenic model systems since the 1990s due to their detection in diseased tissue samples (mRNA and protein)(Kanzler et al. 1999; Tsushima et al. 1996). In the years since, large-

scale studies on the roles of TGFβ signaling began, several roles have been identified for this pathway in a diverse array of contexts including development(Kitisin et al. 2007; Jamora et al. 2004), homeostasis(Clayton et al. 2020; Foitzik et al. 2000; Kubiczkova et al. 2012), wound healing(Ramirez, Patel, and Pastar 2014), and disease(Kanzler et al. 1999), especially cancer(Baba et al. 2022; Gu and Feng 2018; Massagué 2008; Thakur et al. 2020; Tsushima et al. 1996; M. Zhang et al. 2021; Neuzillet et al. 2014); as a result, factors which work to regulate TGFβ signaling are ultimately deeply consequential to the processes it regulates.

In development TGFβ signaling is considered highly consequential and has been found to be evolutionarily conserved in metazoans(Huminiecki et al. 2009). Beginning in embryohood TGFβ family member, Nodal, is indispensable in vertebrate gastrulation as well as mesoderm and endoderm induction. Moreover, Nodal and BMP signaling have also been proven to contribute to dorso-ventral patterning as well as left-right patterning in the vertebrate blastoderm(Shen 2007; Munir et al. 2004; Floc'hlay et al. 2020). Going further, many highly specialized TGFβ family members dictate embryonic organogenesis in vertebrates such as Anti-Müllerian Hormone (AMH) which is required for the regression of Müllerian ducts in male fetuses(M. Y. Wu and Hill 2009); the absence of this hormone or its receptor may lead to persistent Müllerian Duct in males while, interestingly, in females AMH is also required for the proper development of ovarian follicles(M. Y. Wu and Hill 2009). Also notable is the contribution of TGFβ2/3 signaling to development through its ability to induce the epithelial-mesenchymal-transition (EMT) in rudimentary heart tissues which is essential for endocardial cell migration and ultimately heart valve development(M. Y. Wu and Hill 2009). Relating to the development of the skin appendages, several TGFβ family proteins have been found to hold important roles; BMP5 induces skin placodes to become sweat glands rather than hair follicles (Lu et al. 2016)and *TGFβ2* knockout leads to a 50% reduction in

hair bud formation with remaining *TGFβ2*-null hair buds developing abnormally thereafter(Jamora et al. 2004).

Relating to adult organisms, TGFβ signaling also has many roles where disease progression and homeostasis are concerned. Wound healing is one of the most well-studied homeostatic roles of TGFβ signaling. Within unwounded skin epidermis TGFβ1 mainly contributes to tissue homeostasis by acting as a growth-inhibitory cytokine which arrests the cell cycle at early G1 via SMAD-mediated control regulation of cell-cycle regulators such as *c-myc* (Ramirez, Patel, and Pastar 2014). Mutant mice with constitutively expressed *TGFβ1* driven by the *keratin 1* promotor die soon after birth due to severely decreased epidermal proliferation while those with epidermally ablated *TGFβ2* experience loss of tissue homeostasis due to keratinocyte hyperproliferation(Ramirez, Patel, and Pastar 2014). Although a large amount of information related to the compartmentalization of TGFβ signals in human skin wounds unfortunately remains unclear, it is known that TGFβ signaling is required to promote the formation of granulation tissue during the inflammatory and proliferative phases of wound healing as well as collagen deposition by fibroblasts during the remodeling phase(Ramirez, Patel, and Pastar 2014).

In cancer, TGFβ signaling has been heavily studied and consistently found to have a significant role in the initiation and progression of cancers(Baba et al. 2022; Gu and Feng 2018). Although TGFβ often acts as an antiproliferation cytokine and tumor suppressor in normal cells and early cancer cells by inducing cell cycle arrest and apoptosis, in later stages of cancer when lesions become resistant to the cytostatic effects of TGFβ but remain sensitive to it(Baba et al. 2022; Neuzillet et al. 2014), mature cancer lesions may undergo EMT leading to metastasis and therefore disease progression(Xu, Lamouille, and Derynck 2009). Due to these items, *TGFβ* transcript and protein levels have been heavily studied as a predictive biomarker of cancer outcome

in clinical settings with some groups showing interest in targeting TGFβ signaling therapeutically in cancer patients(Baba et al. 2022; Neuzillet et al. 2014; Roberts 2023; Xu, Lamouille, and Derynck 2009) .

1.3 BRCA Associated Protein 1(BAP1)

1.3.1 Structure and Function of BAP1

As its name suggests, BAP1 was first identified as a result of its association with the product of the well-known, breast cancer susceptibility gene, BRCA1(J. Cao and Yan 2012; L. Cao, Li, and Wu 2021) but, in the time since this discovery it has been shown to interact with other proteins including: BRCA1 Associated RING Domain 1 (BARD1), Forkhead Box K1/2 (FOXK1/2), Ying Yang 1(YY1), and Additional Sex Comb Like 1 and 2 (ASXL1/2)(Cheung and Testa 2017). BAP1 is a nuclear ubiquitin carboxy-terminal hydrolase (deubiquitinating enzyme) (Fig. 4).

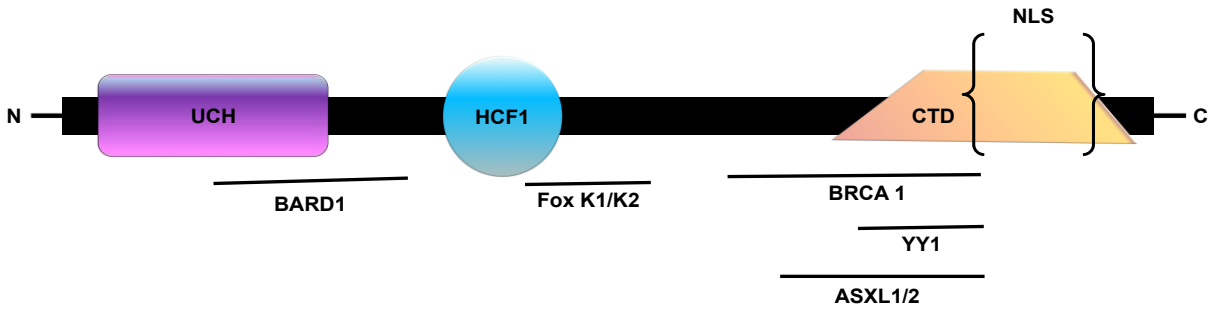

Figure 4. **The Structure of BAP1.** A diagram showing the major domains of BAP1 relative to one another from the N-terminus to the C-terminus. Underlines used to denote the binding regions of known protein interactors. Note: UCH= Ubiquitin C-terminal Hydrolase, HCF1= Host Cell Factor 1, CTD = C-terminal Domain, NLS = Nuclear Localization Signal, BARD1 = BRCA1 Associated RING Domain 1, Fox K1/K2= Forkhead Box Protein K1/K2, BRCA1 = Breast Cancer gene 1, YY1= Ying Yang 1, ASXL1= Additional Sex Combs Like 1/2 .

The binding domains BAP1 contains maintains the structure and function of PR-DUB complexes 1-3 through non-covalent interactions with co-component proteins(Hong et al. 2020). Domains contained within BAP1 include the Ubiquitin Carboxyl-Terminal Hydrolase (UCH) Domain, Host Cell Factor Binding Domain (HBM) and the C-Terminal Domain (CTD) which is followed immediately by a nuclear localization signal which ensures accurate segregation to the nuclear compartment(Hong et al. 2020; Louie and Kurzrock 2020).

1.3.2 Ubiquitin Carboxyl-Terminal Hydrolase Domain

Ubiquitination, in the context of histones, is a form of post-translational modification deposited by E3 ubiquitin ligases which participates in the modulation of the transcription of associated DNA(J. Cao and Yan 2012). Although generally considered a repressive histone mark, histone tail ubiquitination has proven to have diverse effects depending on which octamer subunit

it is associated with; specifically, when associated with histone subunit H2A ubiquitin acts as a highly repressive mark(Bonnet et al. 2022; Wang et al. 2004; Hosseini and Minucci 2018) while when associated with histone H2B it is an activating mark(J. Cao and Yan 2012; L. Cao, Li, and Wu 2021; Hosseini and Minucci 2018). Monoubiquitylation is considered extremely significant to gene regulation, and, in fact, it is believed that histone subunits H2A and H2B are two of the most abundant ubiquitinated proteins with current estimates stating that the proportion of these proteins conjugated to ubiquitin are between 5-15% and 1-2%, respectively(J. Cao and Yan 2012). To properly regulate ubiquitin-mediated transcriptional regulation a number of evolutionarily conserved proteins work in concert to catalyze the addition and removal of these groups such as those which form Polycomb Repressive Complexes (PRC) 1 ,2, and 3(Kolovos et al. 2020; Bonnet et al. 2022; L. Cao, Li, and Wu 2021)

At the N-terminus of BAP1 is a 240 amino acid, highly conserved N-terminal catalytic domain typical of proteins in the UCH family(Bhattacharya, Hanpude, and Maiti 2015; Szczepanski and Wang 2021). This catalytic domain confers BAP1's most studied function, that being a deubiquitinase whose primary substrate is lysine 119 of histone octamer subunit H2A when associated with the Polycomb Repressive Complex 1 (PRC1) to form the Polycomb Repressive Deubiquitinase (PR-DUB)(Fursova et al. 2021; Cheung and Testa 2017). Consequently, this translates to BAP1's ability to hydrolyze mono-ubiquityl conjugates holding an immense and diverse power to modulate gene expression and, in turn, guide several processes related to cell molecular economy including cell proliferation, apoptosis, ferroptosis, immunological response, response to DNA damage, and cell cycle control(Yilei Zhang et al. 2018; Yilei Zhang, Zhuang, and Gan 2019; Louie and Kurzrock 2020; Machida et al. 2009; Szczepanski and Wang 2021). Regarding histone octamer H2A, it was determined to be a key BAP1 deubiquitylation target as

of the 2010s with the characterization of the PR-DUB in which BAP1 minimally complexes in concert with ASXL1/2 and FOXK1/2(Scheuermann et al. 2010; Szczepanski and Wang 2021; Kolovos et al. 2020). Like its Drosophila ortholog, calypso, complexed BAP1 binds established Polycomb group (PcG) targets where it can remove monoubiquitin groups from H2A but not H2B residues(Scheuermann et al. 2010). BAP1's preference for H2AK119Ub is informed by structural elements which are highly conserved among C-terminal deubiquitinases. According to a 2023 work by Thomas and associates, BAP1 interacts with the nucleosome acidic patch using regions unique to it while, in tandem, ASXL interacts with DNA near the nucleosome-DNA exit forming a clamp which in-turn interacts with DNA near the nucleosome dyad(Thomas et al. 2023). In this model, a solved cryo-EM structure of the BAP1/ASXL-nucleosome complex elucidates the role and modality of the disruption to activity caused by the clinically significant BAP1 catalytic activity mutation site C91(Thomas et al. 2023). Ultimately, it was determined that the residues involved in the recognition of the nucleosome substrate are also strictly required for efficient deubiquitylation in vivo(Thomas et al. 2023).

1.3.3 Host Cell Factor Binding Domain

Host cell factor 1 is a heterodimeric protein containing subunits HCF-1N and HCF-1C with known influences in cell cycle regulation(Machida et al. 2009; Yu et al. 2010). This protein is one with high relevance to the known responsibilities of BAP1 due to having a direct association with it in each PR-DUB 1, 2, and 3 via the HCFBD11/6/2023 8:51:00 PM. Within the context of the roles of BAP1, it has been shown that HCF1 is extremely unique in that it is one of the few characterized non-histone targets of BAP1's deubiquitinase activity aside from BAP1 itself(Yu et al. 2010; Machida et al. 2009; Mashtalir et al. 2014). The site of this BAP1-mediated

deubiquitylation is at HCF-1N at K48 of its Kelch domain where conjugated polyubiquitin chains mainly trigger degradation when undisturbed(Machida et al. 2009).

Notably, HCF-1 deubiquitination via BAP1 is required for BAP1-mediated growth regulation and , in addition, growth suppression by dominant negative BAP1 mutants is entirely dependent on the HCF-1 binding motif(Machida et al. 2009). Sustained interaction with HCF-1 by BAP1 takes place via residues within the HCF-1 binding domain whereby the HCF-1 binding motif of BAP1 interacts with the HCF-1N subunit(Yu et al. 2010; Machida et al. 2009). The interaction of these two proteins via the BAP1 HCFBD plays a secondary role in aiding in the initial assembly and stabilization of the trimeric complex between BAP1, HCF-1, and YY1 found within the PR-DUB(Mashtalir et al. 2014).

1.3.4 C-Terminal Domain

At the C-terminus of BAP1 from amino acid 640-710 is the homology C-terminal UCHL5/UCH37-like domain (ULD), this is directly followed by the nuclear localization signal (NLS) and the nucleosome binding domain which aids in recruitment to substrate nucleosomes(Bhattacharya, Hanpude, and Maiti 2015; Hong et al. 2020). This domain is believed to be important for the assembly and stabilization of complexes BAP1 is involved in due to it containing interaction sites for BRCA1, ASXL 1,2, and 3, and YY1(Hong et al. 2020). The BRCA1 interaction interface contained within the C-terminal domain binds to the N-terminal RING domain of BRCA1 ultimately regulating BRCA1-mediated tumor suppressor functions(Bhattacharya, Hanpude, and Maiti 2015; Wang et al. 2004). Interestingly, based on a 2015 work by Bhattacharya et al., it seems that it may also be possible that BAP1's C-terminal

domain serves an auto regulatory function because higher enzymatic activity was observed in truncated mutants than full-length BAP1(Bhattacharya, Hanpude, and Maiti 2015).

1.3.5 BAP1 in Cancer

The effects of BAP1 activity are well known in terms of its relationship to cancer(Cheung and Testa 2017). BAP1 is a bonified tumor suppressor, meaning that its dysfunction in vivo leads to carcinogenesis(Cheung and Testa 2017; Kadariya et al. 2016; Masclef et al. 2021); this phenomenon is due to the altered activity of proteins normally regulated by BAP1 activity following its mutation, generally following a two-hit mechanism(Masclef et al. 2021). In many cases *BAP1* mutations in clinical samples are clustered around the UCH or nuclear localization signal; these mutations tend to negatively impact the accurate segregation of BAP1 to the nuclear compartment or reductions to its catalytic activity(Cheung and Testa 2017; Harbour et al. 2010). Most notable of these is the mutation of site C91 within the catalytic domain(Harbour et al. 2010). In humans, mutations in the *BAP1* gene lead to several cancers and tumor predisposition syndrome, an inherited condition which increases the risk of a variety of malignant and non-malignant tumors(Testa et al. 2011).

Documented cases of at least two families with germline *BAP1* mutations who experience an extremely elevated incidence of rare cancers have been reported in literature. Of note is the relationship between these familial *BAP1* mutations and the development of malignant mesothelioma (MM), an aggressive cancer of the serosal membranes(Cheung and Testa 2017; Testa et al. 2011). In the case of both families MM clustering with a higher rate than those seen in unrelated individuals who reported similar asbestos exposures(Testa et al. 2011). When sequenced, 2 of the 26 assayed individuals with germline *BAP1* mutations showed evidence of

deletions which resulted in a premature stop codon ahead of the NLS; interestingly, both individuals were also previously treated for UM between 1 and 6 years before their MM diagnosis while remaining individuals had no history of UM(Testa et al. 2011). Tumor samples collected from this family cohort revealed a ~22% prevalence of *BAP1* truncation mutants and an ~58% prevalence of total *BAP1* expression loss when cultured(Testa et al. 2011).

In addition to this, Cre-recombinase induced *BAP1* ablation in mice has been shown to lead to cancer very reproducibly(Testa et al. 2011; Kadariya et al. 2016; Yilei Zhang et al. 2018; Yilei Zhang, Zhuang, and Gan 2019). Like human counter parts, *BAP1*-deficinet mice have a 2-fold increased risk of developing MM following even relatively low doses of crocidolite asbestos as well as decreased post-exposure survival(Kadariya et al. 2016). Moreover, at least three different *BAP1* KO mice lines , including two containing clinical *BAP1* mutant knock-ins, are prone to develop spontaneous malignant tumors with a similar spectrum of neoplasms(Kadariya et al. 2016).

1.3.6 The Significance of BAP1 in the Skin

Due to the lack of reports on the roles of BAP1 within the skin, the true nature of BAP1's range of functionality is ripe for exploration with some existing research even indicating that certain cancers of the skin are linked to *BAP1* loss(Masoomian, Shields, and Shields 2018). Specifically, the effects of its activities on items such as the homeostasis, structure, ubiquitination landscape, and distribution of cell populations within the skin are in need of further investigation. Concerning the latter specifically, it would also be interesting to examine the effect of BAP1 on the development of skin appendages, namely the eccrine sweat glands and hair follicles.

In this work various properties of *BAP1*, as it relates to its function in the skin, were examined. As a means of better understanding these items, a strain of knockout (KO) mice which lack a functional *BAP1* protein will be used as an *in vivo* model system. The design of this knockout (KO) includes a floxed first exon as well as Cre-recombinase under the control of a *keratin 14* (*Krt14*) promoter such that the knockout will only take place in tissues with $Krt14^{+}$ progenitors (e.g,. epidermal tissues). Through a thorough examination of the phenotypes of both KO cells and mice, the exact utility, and abilities of *BAP1* in murine skin were investigated.

Chapter 2: The Roles of BAP1 in the Hair Cycle

2.1 Abstract

As a skin appendage organ, the hair follicle provides an excellent paradigm to study tissue homeostasis as adult hair follicles continuously undergo cycles of growth (anagen), regression (catagen), and rest (telogen). BRCA-associated protein 1 (BAP1) is a tumor suppressing de-ubiquitinase best known for its roles in the polycomb repressive complex and hereditary malignant mesothelioma predisposition. To explore its potential role in skin development, we developed a conditional knockout (KO) mouse model of *BAP1*. Tissue-specific loss of *BAP1* in skin epithelial cells driven by *Krt14-Cre* does not significantly alter epidermal stratification or skin wound repair in vivo. However, *BAP1* loss in the skin leads to significant alopecia and altered hair cycle progression in adult skin. In determining the underlying molecular mechanisms, we found that *BAP1* ablation inhibits TGFβ signaling and catagen entry in vivo. Using primary murine keratinocytes isolated from *BAP1* KO skin, our study further indicates that *BAP1* plays a critical role in TGFβ signal transduction by protecting phosphorylated SMAD2 from proteosome-mediated degradation through BAP1's de-ubiquitinase activity. Together, our findings identify an important molecular mechanism underlying the hair cycle and present valuable insight into the roles of BAP1 in skin tissue homeostasis and TGFβ signaling.

2.2 Introduction

Mammalian skin provides an essential barrier that protects us from various environmental damages(Elaine Fuchs 2016). A pilosebaceous unit of mammalian skin consists of a hair follicle, interfollicular epidermis and sebaceous gland. Hair follicles originate from the epidermal stem cells during hair follicle morphogenesis in the embryonic skin(Blanpain et al. 2004; E. Fuchs

2008). In adult animals, the somatic stem cells of the hair follicle reside in a specific niche called the bulge located at the upper portion of the hair follicle. Quiescent bulge stem cells become activated at the start of each hair cycle, allowing the hair follicles to continuously cycle through the defined stages of anagen (active growth), catagen (regression phase) and telogen (rest phase)(Quist and Quist 2021). Both processes, hair follicle morphogenesis and hair cycles are regulated by intricate pathways, including the Wingless (Wnt), Sonic Hedgehog (SHH), and Transforming growth factor β superfamily (TGFβ and bone morphogenic protein, BMP) signaling networks(Daszczuk et al. 2020; Li and Tumbar 2021; Yi 2017). Aberrant regulation of these pathways leads to various developmental disorders and skin diseases.

Breast Cancer (BRCA)-associated Protein 1 (BAP1) was identified as a ubiquitin carboxy-terminal hydrolase that binds to the RING finger domain of the Breast Cancer type 1 susceptibility protein (BRCA1)(Cheung and Testa 2017; Masclef et al. 2021). The BAP1 protein is comprised of an N-terminal ubiquitin carboxyl terminal hydrolase domain (UCH), an HCF1 binding domain(HCFBD) , and a C-terminal domain containing the BRCA1 binding site, Yin Yang 1 (YY1) binding region as well as an ASXL1/2 binding domain. The N-terminal ubiquitin hydrolase domain confers BAP1's ability to remove ubiquityl groups from target molecules, such as histone H2A tails. BAP1 is a subcomponent of the Polycomb repressive de-ubiquitinase complex (PR-DUB) that antagonizes the activity of the Polycomb repressive complex 1 (PRC1), which catalyzes H2A ubiquitination at Lysine 119 through the RING1A/B E3 ligase(De Napoles et al. 2004; Scheuermann et al. 2010; Wang et al. 2004). Germline *BAP1* mutations that compromise this de-ubiquitinase activity are associated with the development of various human cancers, such as mesothelioma(Testa et al. 2011), uveal melanoma(Harbour et al. 2010), renal cell carcinoma(Peña-Llopis 2012), and cholangiocarcinoma(Jiao 2019). Consistent with its role as a tumor suppressor,

BAP1 has also been implicated in various cellular processes including: cell cycle regulation, DNA damage repair, chromatin modification, and modulation of metabolic stress (Masclef et al. 2021; Cheung and Testa 2017).

Despite our current knowledge of BAP1 in the PR-DUB and tumorigenesis, much remains to be learned about its potential role in development and adult tissue homeostasis. Using a mouse genetics approach, we found that the ablation of *BAP1* in skin epithelial cells does not affect epidermal differentiation or skin wound healing but leads to alopecia in adult animals and aberrant hair cycle progression in vivo. Histology revealed a striking defect of *BAP1* KO hair follicles at catagen entry during the hair cycle in adult animals. Catagen is the transition stage between anagen and telogen, during which, the lower segment of the hair follicle undergoes a dramatic regression through apoptosis of the epithelial cells in the bulb and inner root sheath(Oh et al. 2016). Though the precise mechanisms that drive the transition from anagen to catagen remain largely elusive, it is believed that the matrix cells in the bulb region act as transit-amplifying cells with limited proliferative potential(Chen et al. 2020; Alonso and Fuchs 2006) and that as the supply of new matrix cells declines, the differentiation process in the hair shaft is inhibited and catagen entry is triggered. Previous studies suggest that this transition is regulated by SGK3 and Msx2, which inhibit catagen entry and maintain the anagen phase of hair follicles(Alonso and Fuchs 2006). By contrast, TGFβ family proteins, such as TGFβ1 and BMPRIa, promote catagen entry in vivo (Foitzik et al. 2000; Jamora et al. 2004; Oshimori and Fuchs 2012)

Due to the strength and consistency of the epidermal *BAP1* KO phenotype in vivo, pursuing the exact causes was highly indicated. Primary keratinocytes were isolated from p0 *BAP1* KO pups and their WT littermates so TGFβ signaling could be interrogated in a purified cell culture. Upon examining the secondary messenger output of *BAP1* KO cells following exposure to

recombinant TGFβ1, we found that *BAP1* deletion can enhance the ubiquitination of phosphorylated SMAD2 and abolish TGFβ signal transduction *in vitro* and *in vivo*. Protein ubiquitination is a universal mechanism for proteosome-mediated protein degradation which regulates a wide variety of cellular processes, including the TGFβ signaling pathway(Imamura, Oshima, and Hikita 2013; Iyengar 2017). Our data show that BAP1 can act as a deubiquitinating enzyme for SMAD2, which requires its N-terminal ubiquitin hydrolase domain. Taken together, our results illuminate an important molecular mechanism whereby TGFβ signal transduction and, in turn, the hair cycle are regulated by BAP1 in murine skin.

2.3 Materials and Methods

Antibodies and Reagents

Mouse and rabbit β actin antibodies (66009-I-Ig and 20536-I-AP, respectively) were obtained from Proteintech (Rosemont, IL). Rabbit Krt 14 and Krt 10 antibodies were obtained from Covance (Princeton, NJ). Rabbit antibody against phospho SMAD2 (138D4) and total SMAD2/3 (3102S) were obtained from Cell Signal (Danvers, MA). Rat anti total TGFβr1 and mouse anti total TGFβr2 were obtained from Santa Cruz Biotechnology. Rabbit anti BAP1 antibody (A302-242A) was obtained from Bethyl Laboratories.inc (Montgomery, TX). Other chemicals or reagents were obtained from Sigma, unless otherwise indicated.

Generation of Skin-Specific *BAP1* KO Mice

The *BAP1* fl/fl line were graciously provided by Dr. Boyi Gan (Dai et al., 2017) at The University of Texas, MD Anderson Cancer Center. These founders were bred with our lab's *K14-Cre* transgenic mice to generate skin conditional *BAP1* KO. To avoid maternal effect phenotypes due

to *K14* activity in the oocyte, breeding pairs only consisted of *K14-Cre*$^{-}$ dams and *K14-Cre*$^{+}$ sires. All mice used in this study were bred and maintained at the ARC (animal resource center) of the University of Chicago in accordance with institutional guidelines and fed on standard chow.

Histology and Immunofluorescence

Skin or wound samples were embedded in OCT over dry ice or fixed in 4% paraformaldehyde then embedded in paraffin and cut into 5μm sections. Sections were subjected to hematoxylin and eosin staining, immunohistochemical staining, or immunofluorescence staining as described (Guasch et al., 2007). Antibodies were diluted according to manufacturer's instruction unless indicated. Measurements of stratification markers were carried out using ImageJ.

Skin Wound Healing

For skin wound healing assays, littermates of ~ 12 wk old mice were anesthetized and had their backs depilated using Nair. Six equidistant, full-thickness excisional wounds were made on both sides of the dorsal midline(X. Wu, Kodama, and Fuchs 2008). Mice were housed separately, and no self-induced trauma was observed in control or cKO mice. Tissue was then examined every day for 8 days after wounding, and wound re-epithelialization was evaluated by both ImageJ tools and histological analyses. Hyperproliferative epidermis was identified by hematoxylin and eosin staining, and the length of HE that extended into the wounds was measured.

2.4 Results

2.4.1 Conditional Ablation of *BAP1* Does Not Affect Epidermal Stratification or Wound Healing

To investigate the role of BAP1 in skin development and tissue homeostasis, we generated

a *BAP1* skin knockout (KO) mouse model. Via homologous recombination, a targeting cassette containing loxP sites was inserted into the *BAP1* locus in mouse chromosome 14(Dai et al. 2017). To target *BAP1* in skin cells with epithelial lineage, we bred $BAP1^{fl/fl}$ mice with *K14*-Cre recombinase transgenic mice, which efficiently excised exon 4 of *BAP1* by embryonic day E15.5 (Vasioukhin et al. 1999) (Fig. 5A and Supplementary Fig. 1A). Neonatal mice genotypic for *K14*-Cre and $BAP1^{fl/fl}$ alleles (KO) are born in the expected Mendelian numbers and grow to normal size as adults. Immunoblotting confirms the loss of *BAP1* in the skin epithelial cells of KO animals (Fig. 5B). Histological analyses of newborn skin shows normal epidermal differentiation despite the loss of *BAP1*; *BAP1* KO skin contains all three differentiated epidermal layers, including the spinous, granular, and stratum corneum layers, resembling WT skin (Fig. 5C). Immunofluorescence with antibodies against keratin 5 (basal layer) or keratin10 (spinous layer) displayed localization patterns analogous to those seen in WT skin (Fig. 5 D and E). Skin wounding can mobilize quiescent epidermal progenitor cells for proliferation and migration. When challenged with full-thickness wounds, both WT and BAP1 KO skin exhibit similar wound closure (Supplementary Fig. 2). Together, these results suggest that *BAP1* is dispensable for epidermal stratification and skin wound repair *in vivo*.

2.4.2 Skin KO of *BAP1* Leads to Hair Loss and Aberrant Hair Cycle in Adult Mice

Although *BAP1* loss does not change skin tissue architecture in young animals, KO animals exhibit significant alopecia as they age (Fig. 6A). By 12 weeks, the KO animals usually have a visibly thinner hair coat compared to their WT littermates, particularly in the ventral skin. Histological analysis of adult skin samples reveal significantly reduced hair follicle density in KO mice (Fig. 6B and quantification in 6C).

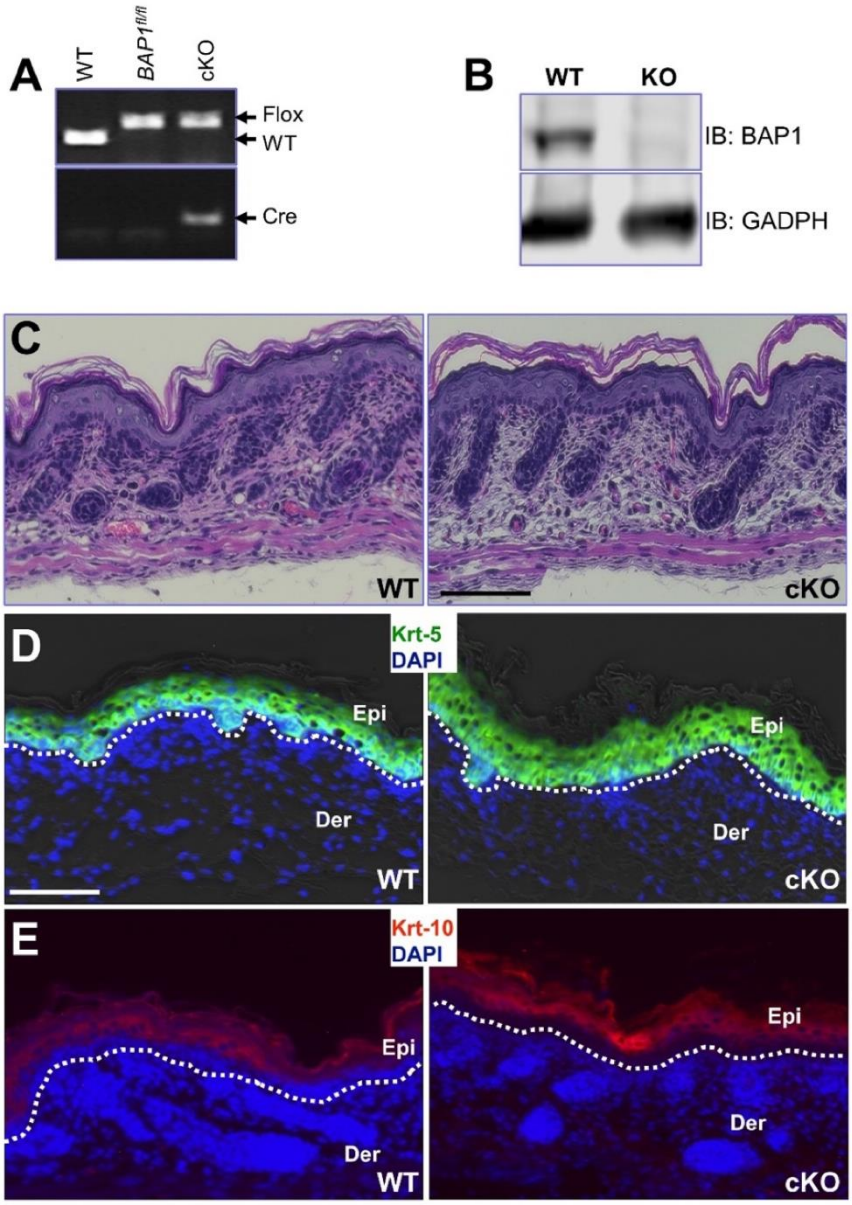

Figure 5. **The Development of a Skin *BAP1* cKO Animal Model.** (A) Typical PCR genotyping results for WT, *BAP1* floxed, and cKO mice. (B) Immunoblots (IB) confirm loss of *BAP1* in KO keratinocytes. (C) H/E staining of newborn skin sections from WT and *BAP1* cKO mice. Scale bar = 100 μm. (D-E) Newborn skin section from WT or *BAP1* cKO animals were stained with antibody against Krt-5 (keratin 5) (D) or Krt-10 (E). Tissue samples were counterstained with DAPI to visualize cell nucleus. Dashed lines denote boundary of dermis (Der) and epidermis (Epi). Scale bar = 100 μm.

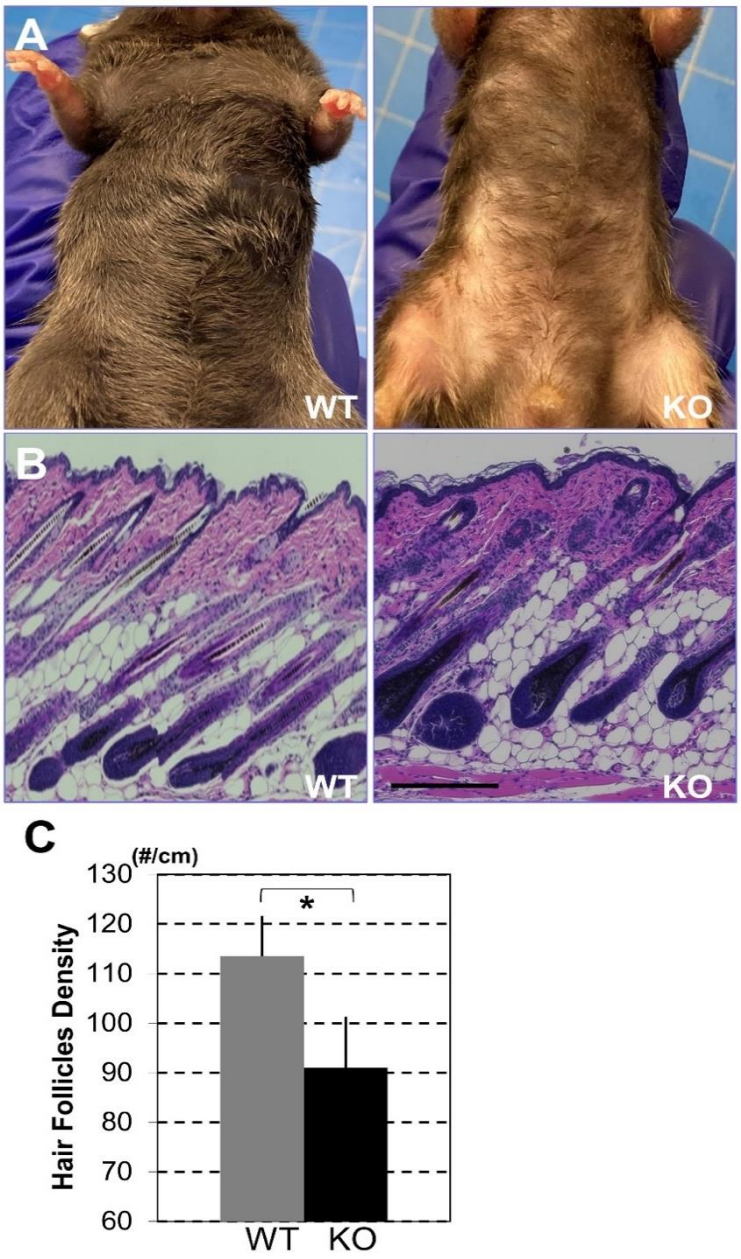

Figure 6. **Loss of *BAP1* Leads to Alopecia in Adult Mice**. (A) Hair loss at the ventral skin in *BAP1* cKO animals (12 weeks old). (B) H/E staining of skin sections from adult (12 weeks old) WT or *BAP1* cKO animals demonstrates reduced hair follicles upon loss of *BAP1*. Scale bar = 100 µm. (C) Bar graph demonstrates reduced hair follicle density (number of follicles per centimeter). n=3 each, *: P<0.05 (Student's t-test).

As hair follicle morphogenesis is not significantly altered in KO skin (Fig. 7A), our data strongly suggest that BAP1 may regulate hair cycles in adult skin.

To characterize the changes in hair cycle upon *BAP1* loss, we next collected skin samples at different ages for histological analysis(Alonso and Fuchs 2006; Oh et al. 2016). Hair follicles in WT and KO skins display similar length and architecture at P9, consistent with normal hair follicle morphogenesis *in vivo* (Fig. 7A). However, histology demonstrates that while the first catagen is fully underway for the WT animals at P17, the KO hair follicles still exhibit an anagen phenotype including highly pigmented hair shafts and elongated structure. By P20 when the WT hair follicles reach the telogen phase as expected, KO skin contains a mixed population of hair follicles with both anagen and catagen-like morphology (Fig 7A).

Catagen entry is accompanied by the inhibition of transit amplifying cell proliferation in the hair follicle (Oh et al. 2016). The aberrant catagen entry seen in KO hair follicles is made more apparent when skin samples are stained for Ki67. Both WT and KO hair follicles exhibit robust cell proliferation at P9 with $Ki67^+$ matrix cells within the bulb, consistent with their full anagen stage. The Ki67 staining is drastically reduced in WT hair follicles at P17. By contrast, most *BAP1* KO hair follicles retain proliferative, $Ki67^+$ matrix cells in the bulb at P17, suggesting elongated anagen and impaired catagen entry secondary to *BAP1* loss (Fig. 7B).

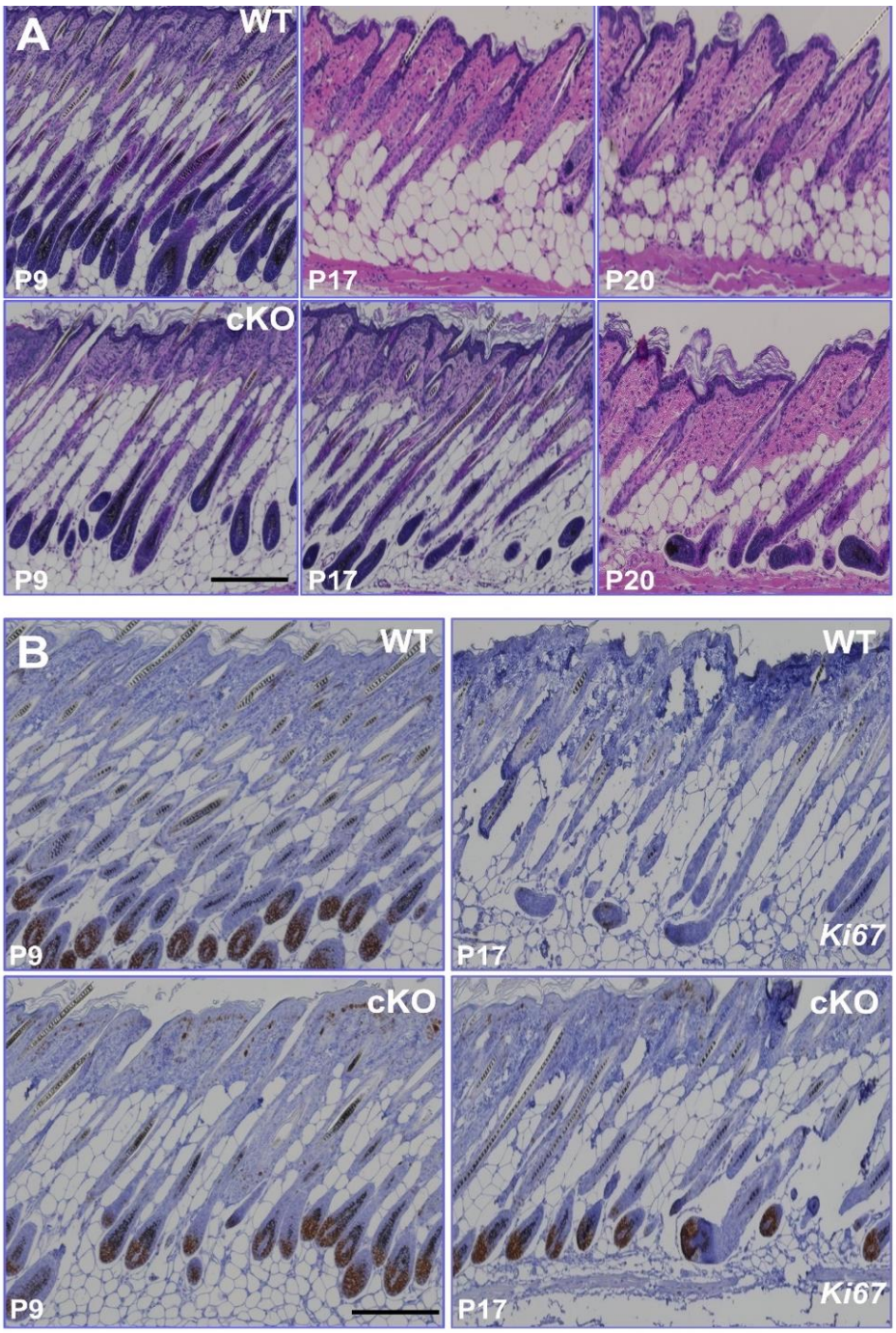

Figure 7. **BAP1 Loss Impairs Catagen Entry During the Hair Cycle.** (A) H/E staining of skin sections from WT or *BAP1* cKO mice during hair follicle morphogenesis (p9), first catagen (p17), and telogen (p20). Scale bar = 100 µm. (B) Immunohistochemistry staining of WT or *BAP1* cKO skin at P9 or P17 with antibody against Ki67. Scale bar = 100 µm

2.4.3 BAP1 Regulates TGFβ Signaling in Skin

To explore the underlying molecular mechanisms, we examined three groups of factors previously identified as important regulators of hair follicle growth, development, and homeostasis: the TGFβ superfamily (TGFβ and BMP), Wnt, and SHH pathways(Daszczuk et al. 2020; Li and Tumbar 2021; Yi 2017). Interestingly, immunohistochemical staining of skin samples near catagen entry (P14) demonstrate remarkable loss of phospho SMAD2 staining in *BAP1* KO animals (Fig. 8), suggesting *BAP1* loss abrogates TGFβ signal transduction in hair follicles. Interestingly, KO hair follicles exhibit more intense staining for phospho-SMAD1/5/8 (BMP signaling), non-phospho β-catenin (Wnt signaling), and Gli1 (SHH pathway) through the length of the IRS than WT counterparts which only exhibit limited staining in the lower aspect of the hair bulb; this suggests that *BAP1* deletion results in a more anagen-like phenotype at later stages *in vivo* (Fig. 4).

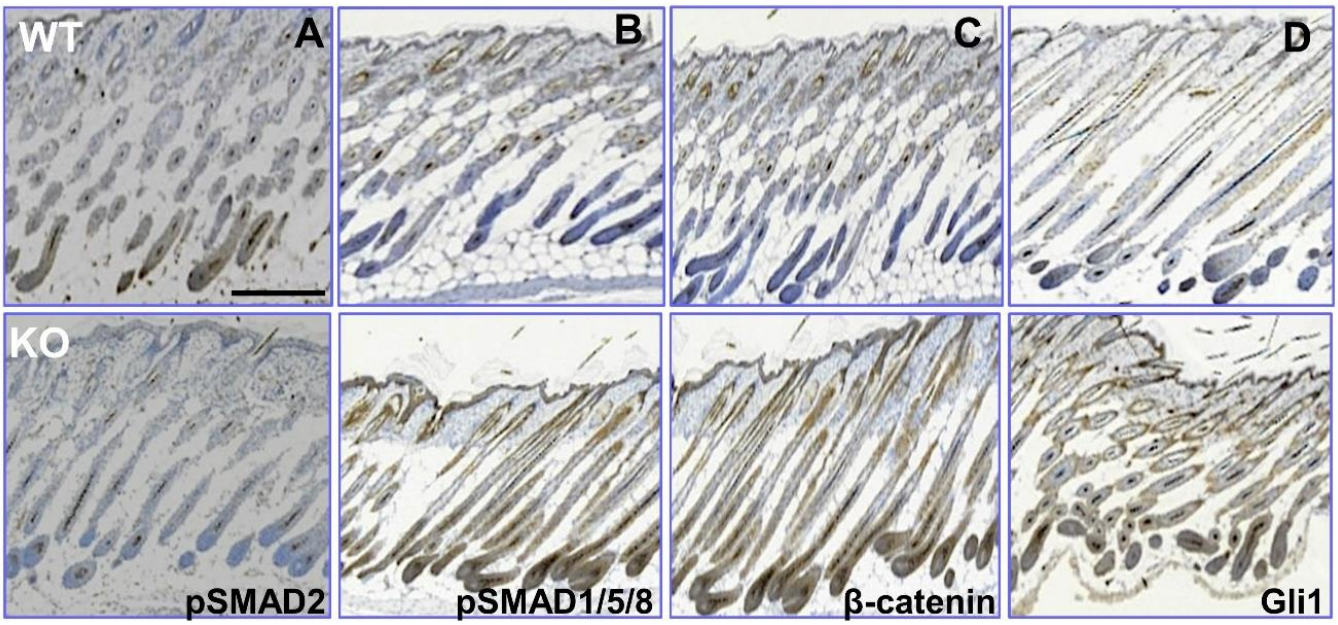

Figure 8. **BAP1 Loss Leads to Decreased TGFβ Signaling *in vivo*.** Immunohistochemistry staining of WT or *BAP1* cKO mouse skin at P14 (before catagen entry) with antibodies against phospho-SMAD2 (TGFβ signaling), phospho-SMAD1/5/8 (BMP signaling), non-phospho β-catenin (Wnt signaling), or Gli1 (SHH signaling). Scale bar = 100 μm.

Chapter 3: The Roles of BAP1 in TGFβ Signaling

3.1 Materials and Methods

Antibodies, Reagents, and Plasmid DNA Constructions

Mouse and rabbit β actin antibodies (66009-I-Ig and 20536-I-AP, respectively) were obtained from Proteintech (Rosemont, IL). Rabbit Krt 14 and Krt 10 antibodies were obtained from Covance (Princeton, NJ). Rabbit antibody against phospho SMAD2 (138D4) and total SMAD2/3 (3102S) were obtained from Cell Signal (Danvers, MA). Rat anti total TGFβr1 and mouse anti total TGFβr2 were obtained from Santa Cruz Biotechnology. Rabbit anti BAP1 antibody (A302-242A) was obtained from Bethyl Laboratories.inc (Montgomery, TX). Other chemicals or reagents were obtained from Sigma, unless otherwise indicated.

Cell Culture

Primary mouse keratinocytes were isolated from the epidermis of newborn mice using trypsin, after prior separation of the epidermis from the dermis by an overnight dispase treatment. Keratinocytes were plated on mitomycin C-treated 3T3 fibroblast feeder cells until passage 3. Cells were cultured in E-media supplemented with 15% serum with a final concentration of 0.05 mM Ca^{2+}.

Protein Biochemical Analysis

Western blot was performed as described previously(Lee et al. 2021). Cell lysates were prepared with RIPA (radioimmunoprecipiation assay) buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% Glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) containing protease inhibitors and phosphatase inhibitors. Equal concentrations of processed cell

lysates were separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a NC membrane. The immunoblot was incubated with Odyssey blocking buffer (Li-Cor) at room temperature for 1 h, followed by an overnight incubation with the primary antibody. Blots were washed three times with 0.01% Tween 20/Tris-buffered saline (TBST) and incubated with a 1:10000 dilution of secondary antibody for 1 h at room temperature. Blots were washed three times with TBST. Visualization and quantification were carried out with the LI-COR Odyssey scanner and software (LI-COR Biosciences, Nebraska, USA).

For TGFβ1 stimulation, recombinant mouse TGFβ1 was resuspended as per the manufacturers' instructions and added to E-media supplemented with 15% serum and 0.05 mM Ca^{2+} to a final concentration of 7.7 nM before being added to Keratinocytes grown to 80% confluency. Cells were allowed to incubate afterward at 37°C with 7.5% CO_2 for 1 or 3 hours then lysed for analysis via Western blotting. For proteasome inhibition, MG-132 (Millipore Sigma) was added to E- low Calcium media to a final concentration of 16 μM. Cells were allowed to incubate afterward at 37°C with 7.5% CO_2 for 2 hours before being exposed to 7.7 nM TGFβ1.

Statistical Analysis

Statistical analysis was performed using Excel software. An average of at least 3 replicates was used to describe the entire population without assumptions on the statistical distribution. A one-tailed Student's t-test was used to assess the statistical significance (P value) of the difference for most experiments.

3.2 Results

3.2.1 *BAP1* Ablation Impairs TGFβ Signalling in Keratinocytes

TGFβ signalling activation is critically involved in the anagen-to-catagen transition(Foitzik et al. 2000; Oshimori and Fuchs 2012). To investigate the role of BAP1 in this pathway, we isolated and cultured primary mouse keratinocytes from both WT and KO P0 skin (Supplementary Fig. 3). Consistent with our findings in vivo, when stimulated with recombinant TGFβ in vitro, the *BAP1* KO keratinocytes displayed markedly reduced SMAD2 phosphorylation compared to WT counterparts (Fig. 9A and quantification in Fig. 9B). The reduction of SMAD2 phosphorylation is not due to changed expression of SMAD2 or TGFβ receptors. Immunoblots with WT and *BAP1* KO keratinocytes confirm similar expression level of SMAD2 and TGFβr1/2 (TGFβ receptor 1 and 2).

To examine TGFβ signal transduction *in vitro*, we used a luciferase reporter that contains three repeats of a 12-o-tetradecanoylphorbol 13-acetate (TPA)-responsive element plus the plasminogen activator inhibitor (PAI) promoter (p3TP-Luciferase reporter)(Wrana et al. 1992). This promoter has been shown to be specifically stimulated by TGFβ to drive luciferase expression. Transfected WT keratinocytes display dramatically elevated reporter activity in vitro upon TGFβ stimulation as expected. *BAP1* loss causes significantly reduced luciferase activity when the same treatment is applied (Fig. 9C). Together, our data provide strong evidence that BAP1 could potentially regulate catagen entry in murine hair follicles by controlling TGFβ signalling in skin.

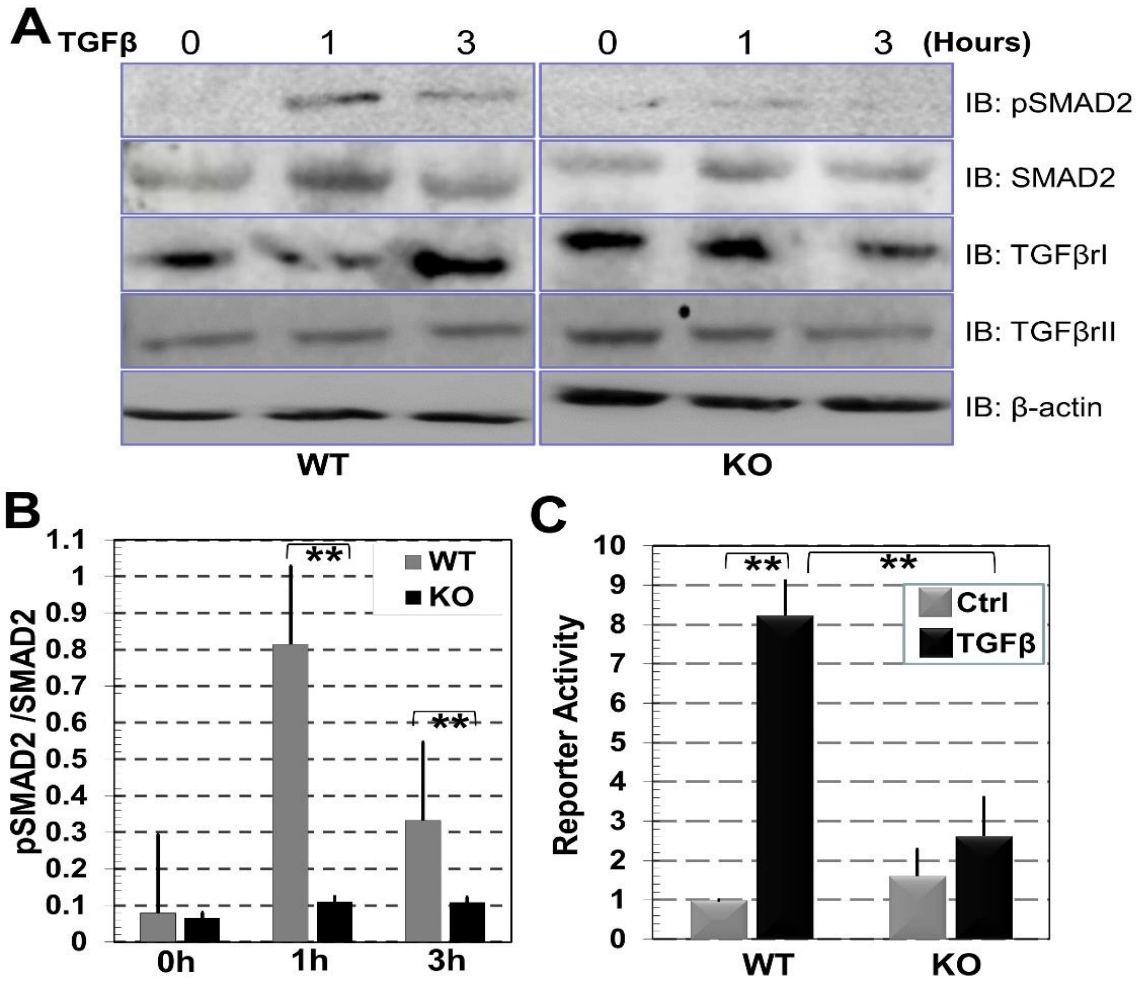

Figure 9. Ablation of BAP1 Impairs TGFβ Signaling *in vitro*. (A) Immunoblots of WT or *BAP1* KO keratinocytes with different antibodies as indicated, upon stimulation with recombinant TGFβ1 *in vitro*. (B) Bar graph for the ratio of phospho-SMAD2/total SMAD2, as determined by densitometry analysis. n = 3 for each sample. **: P<0.01 (Student's t-test). (C) TGFβ1 signal transduction in WT or *BAP1* KO cells was determined by a luciferase reporter (p3tp-Lux) with or without recombinant TGFβ1 stimulation. n = 3 for each sample. **: P<0.01 (Student's t-test).

3.2.2 BAP1 Regulates SMAD2 Ubiquitination Through its De-Ubiquitinase Activity

As a key signal transducer, SMAD2 is phosphorylated by the TGFβr1/2 complex upon ligand engagement and translocated to the nucleus where it influences gene transcription. However, TGFβ signaling also requires precise regulation of activated SMAD2. It has been well documented that phosphorylated and activated SMAD2 is subjected to ubiquitination and proteasome-mediated protein destruction, which provides a key negative feedback mechanism to control TGFβ signaling(Imamura, Oshima, and Hikita 2013; Iyengar 2017).

BAP1 harbors the N-terminal, ubiquitin C-terminal hydrolase domain, which can de-ubiquitinate histone H2A and other target proteins. To investigate the molecular mechanism of BAP1's regulation of TGFβ signal transduction, we first examined whether pharmacological inhibition of proteasome degradation can affect TGFβ signaling in *BAP1* KO cells. Interestingly, immunoblots revealed significantly elevated phospho SMAD2 levels in KO cells upon TGFβ stimulation in the presence of MG132, at a level similar to WT counterparts, suggesting that proteasome inhibition can restore TGFβ signal transduction in *BAP1* null cells (Fig. 10A and quantification in Fig. 10B). Consistent with our data above, neither MG132 treatment nor TGFβ stimulation alter the expression of SMAD2 or TGFβr1/2 (Fig. 10 A).

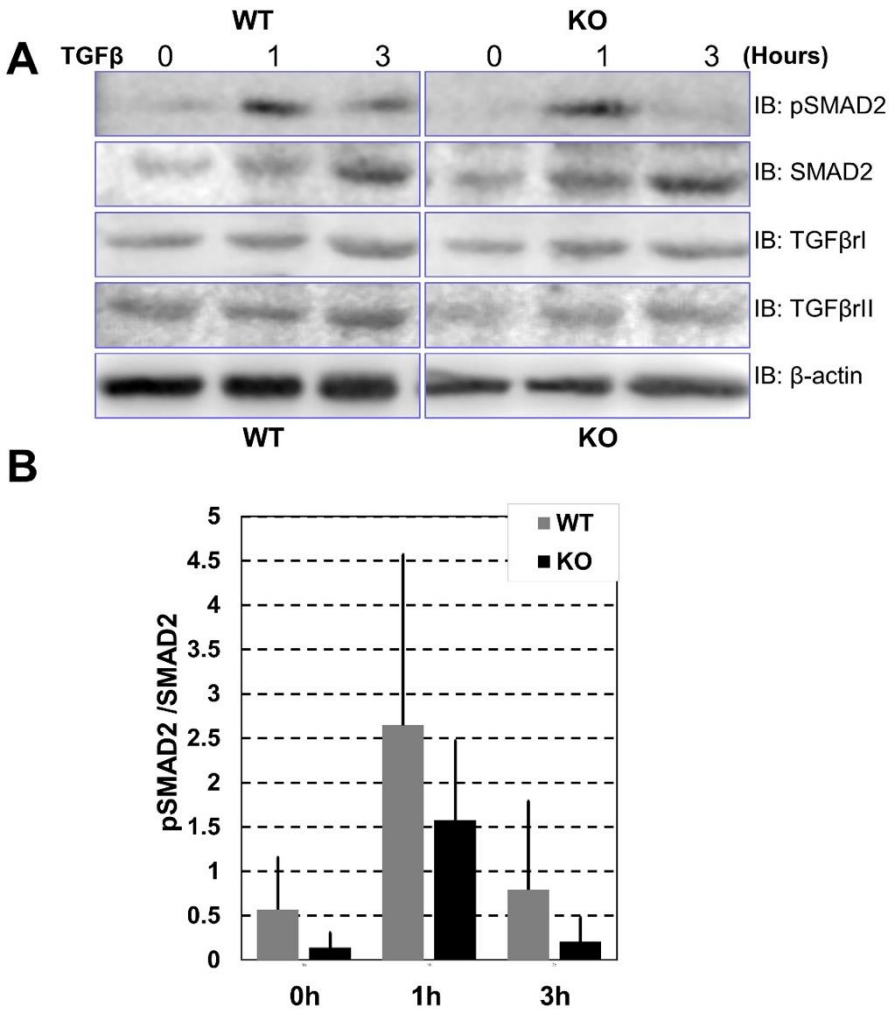

Figure 10. BAP1 Regulates TGFβ Signaling Through Protein Degradation Pathway. (A) Immunoblots of WT or *BAP1* KO keratinocytes with different antibodies as indicated, upon treatment with proteasome inhibitor MG132 and recombinant TGFβ1 in vitro. (B) Bar graph for the ratio of phospho-SMAD2/total SMAD2, as determined by densitometry analysis. n = 3 for each sample.

3.2.3 BAP1 Regulates SMAD2 Ubiquitination Via Its N-Terminal Ubiquitin Hydrolase Activity

To determine whether BAP1 regulates SMAD2 ubiquitination, we co-transfected both WT and *BAP1* KO cells with plasmids encoding Hemagglutinin (HA) tagged SMAD2 and Myc tagged ubiquitin. Immunoprecipitation analysis indicates that SMAD2 ubiquitination is dramatically increased in KO cells when stimulated with TGFβ (Fig 11 A). To confirm that this change is due to the loss of *BAP1*, we carried out rescue experiments by re-expressing WT *BAP1* or *BAP1 C91A* (catalytically inactive mutant) in KO cells. Immunoblots reveal that WT *BAP1* but not the mutant *BAP1* deficient in de-ubiquitinase activity can restore its function to suppress SMAD2 ubiquitination in skin keratinocytes (Fig. 11 A). Consistent with SMAD2 ubiquitination findings, the expression of WT *BAP1* but not *BAP1 C91A* restores TGFβ signal transduction in KO cells as determined by the p3TP-Luciferase reporter assay (Fig. 11 B). Taken together, our results provide compelling evidence that BAP1 regulates TGFβ signal transduction in skin epithelial cells by protecting phosphorylated SMAD2 via its de-ubiquitination activity.

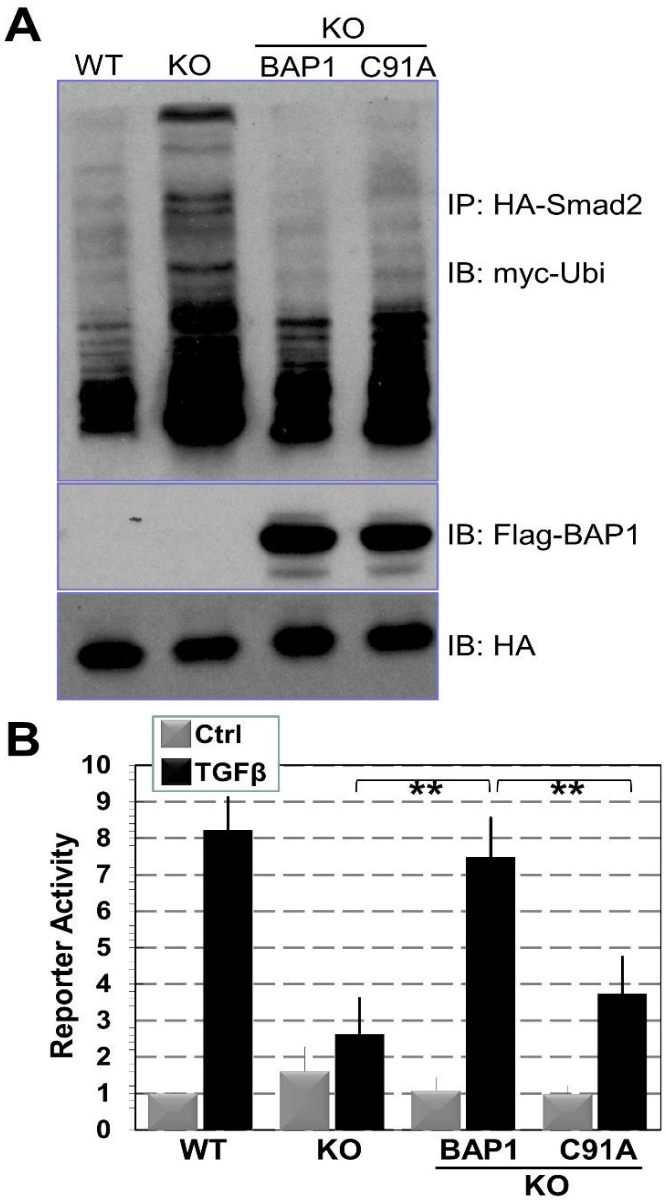

Figure 11. **BAP1 Regulates TGFβ Signaling Through its Ubiquitin Hydrolase Activity**. (A) WT or *BAP1* KO keratinocytes were transfected with plasmids encoding HA-tagged SMAD2 and Myc-tagged ubiquitin. To rescue *BAP1* loss, KO cells were also transfected with plasmids encoding Flag-tagged WT *BAP1 or BAP1 C91A* (mutant deficient in ubiquitin hydrolase activity). Ubiquitination of SMAD2 was determined by immunoprecipitation (IP) with antibody against HA followed with immunoblots with antibody against Myc. Expression of exogenous BAP1 and SMAD2 was also determined by immunoblots with different antibodies as indicated. (B) TGFβ signal transduction in WT, BAP1 KO, or KO cells with rescue expression of *BAP1* or *BAP1 C91A* mutant was determined by a luciferase reporter (p3tp-Lux) upon recombinant TGFβ stimulation. n = 3 for each sample. **: P<0.01 (Student's t-test).

C**hapter 4: Discussion and Future Directions**

4.1 Overview of BAP1 and the Hair Follicle

The hair follicle is a mini-organ native to mammalian skin, which participates in a variety of functions including sensory activities, physical protection, and thermoregulation(Alonso and Fuchs 2006; Daszczuk et al. 2020; Li and Tumbar 2021). As a model system, hair follicles are significant to the fields of stem cell and regenerative biology due to their ability to regenerate themselves in a cyclic, self-regulated fashion, which includes cell death, proliferation, and stasis(Blanpain et al. 2004; E. Fuchs 2008; Elaine Fuchs 2007; 2016). As compared to other stem cell systems, the hair follicle is extremely unique in terms of its accessibility, due to being an external organ, and the relative ease of the *in vitro* culture of its somatic stem cell population. However, despite these advantages, many details of the inner workings of this system and its finer controls are not well understood. In this study, we have identified an important mechanism whereby the ubiquitin hydrolase, BAP1, regulates the signaling of TGFβ to control catagen progression during hair cycle. Although *BAP1* was initially identified as a major tumor suppressor mutated in different human malignancies, accumulating evidences suggest that BAP1 is also critically involved in development, stem/progenitor cell self-renewal, and lineage commitment(Masclef et al. 2021). Conditional ablation of *BAP1* in the hematopoietic lineage impairs the normal differentiation of hematopoietic stem cells (HSC) toward the myeloid lineage and leads to the development of myelodysplastic syndrome(Dey et al. 2012; LaFave et al. 2015). Inactivation of *BAP1* also inhibits the maturation of thymocytes and arrests the progenitor cells at the double-negative 3 stage(Arenzana et al. 2018). The role of *BAP1* in development is evolutionarily conserved; *BAP1* mutations in *Xenopus lavevis* leads to aberrant embryogenesis,

including severe gastrulation defects and developmental abnormalities in ectoderm, mesoderm, and neural crest lineages(Kuznetsov et al. 2019).

4.2 The Interaction of BAP1 and Known Substrates in Our Model.

In our model (Fig. 12), BAP1 stabilizes the key downstream effector of the TGFβ signaling pathway, SMAD2, via its deubiquitinase activity. Ubiquitination plays a critical role in TGFβ signal transduction(Imamura, Oshima, and Hikita 2013; Iyengar 2017). It has been well documented that SMAD proteins, such as SMAD2/3 and 4 can enter the nucleus upon activation and phosphorylation, which can induce expression of the negative regulator, SMAD7. SMAD7 can suppress TGFβ receptor activity and induce its ubiquitination through E3 ligase, SMURF1/2. In addition, phosphorylated SMAD2/3 may also be recognized by Nedd4-L, a different ubiquitin E3 ligase. Together, this provides a robust negative feedback loop to control TGFβ signal transduction. BAP1 contains an N-terminal ubiquitin hydrolase domain. It has been well established that BAP1 is a key component of the PR-DUB, antagonist of the activity of PRC1, by removing ubiquitin groups at Lysine 119 of histone H2A (De Napoles et al. 2004; Scheuermann et al. 2010; Wang et al. 2004). Additionally, BAP1 has been shown to de-ubiquitinate other nuclear proteins, mostly transcription factors, such as YY1, PGC-1α, KLF5, and HCF-1(Machida et al. 2009; Misaghi et al. 2009; Qin et al. 2015; Ruan et al. 2012; Yu et al. 2010).Through these substrates, BAP1 regulates various biological processes, such as cell cycle regulation, mitochondria function, and cell death pathways(Masclef et al. 2021). Our results have identified SMAD2 as a new substrate of BAP1's de-ubiquitination activity, suggesting that BAP1 plays important and diverse functions in cell signaling through its ubiquitin hydrolase activity. Aberrant TGFβ2 signaling in skin has been shown to impair hair follicle development and results in severe hair loss in vivo (Foitzik et al. 2000; Jamora et al. 2004; Oshimori and Fuchs 2012),resembling

the phenotypes of *BAP1* skin cKO mice; as a result, our group asserts that the alopecia seen in *BAP1* cKO mice is likely due to this same mechanism noted by Jamora and associates wherein deficient SMAD signaling caused by *BAP1* loss abrogates TGFβ2 signaling in early hair follicles leading to their development getting arrested.

4.3 The Significance of TGFβ in BAP1-Associated Cancer Initiation and Progression.

BAP1 mutations in humans contribute to the development of various cancers (Masclef et al., 2021), such as mesothelioma (Cheung and Testa 2017; Masclef et al. 2021), uveal melanoma(Harbour et al. 2010), clear cell renal cell carcinoma(Peña-Llopis 2012), thymic epithelial tumor(Wang et al. 2004), and hepatocellular carcinoma(Woo et al. 2017; Mosbeh et al. 2018). Consistent with this notion, TGFβ signaling is considered a tumor suppressor in early carcinogenesis due to its role in restricting cell proliferation(Derynck, Turley, and Akhurst 2021). Loss of TGFβ activity can promote the initiation, progression, and poor clinical outcome of many cancers. In skin, it has been shown that conditional deletion of *TGFβr2* can impair tissue homeostasis and lead to the development of squamous cell carcinoma (SCC)(Guasch et al. 2007). Interestingly, TGFβ signaling also concentrates at the tumor-stroma interface, promoting the heterogeneity of SCC stem cells and drug resistance of skin tumors(Oshimori and Fuchs 2012). Cutaneous SCC is the second most common human cancer, afflicting more than 250,000 patients in the United States every year(Murad and Désirée 2001). Cutaneous SCC can be highly invasive and metastatic (3-10% rate of metastasis), leading to severe morbidity and mortality.

4.4 Future Directions

With this *BAP1* cKO model our group hopes to further explore the role of BAP1 and its regulation of TGFβ signaling in skin carcinogenesis in the future. Amongst the projected future directions are examining the progression and acquisition of skin carcinogenesis *in vivo*. Additionally, the molecular mechanism of BAP1's regulation of TGFβ signaling may also be examined further in the future; herein, the effects of *BAP1* on phospho SMAD accumulation following stimulation with recombinant TGFβ 2 and 3 would be examined. Moreover, the use of a C-terminal *BAP1* mutant in rescues could elucidate whether BAP1 complexation with PR-DUB is strictly required for prolonging activated SMAD2 signaling. Further, it may be interesting to investigate whether BAP1's relationship with BRCA1 is important to the mechanism uncovered in this work.

In closing, our findings provide critical insights into the molecular machinery of the intricate signaling network underlying skin tissue homeostasis and hair cycle progression.

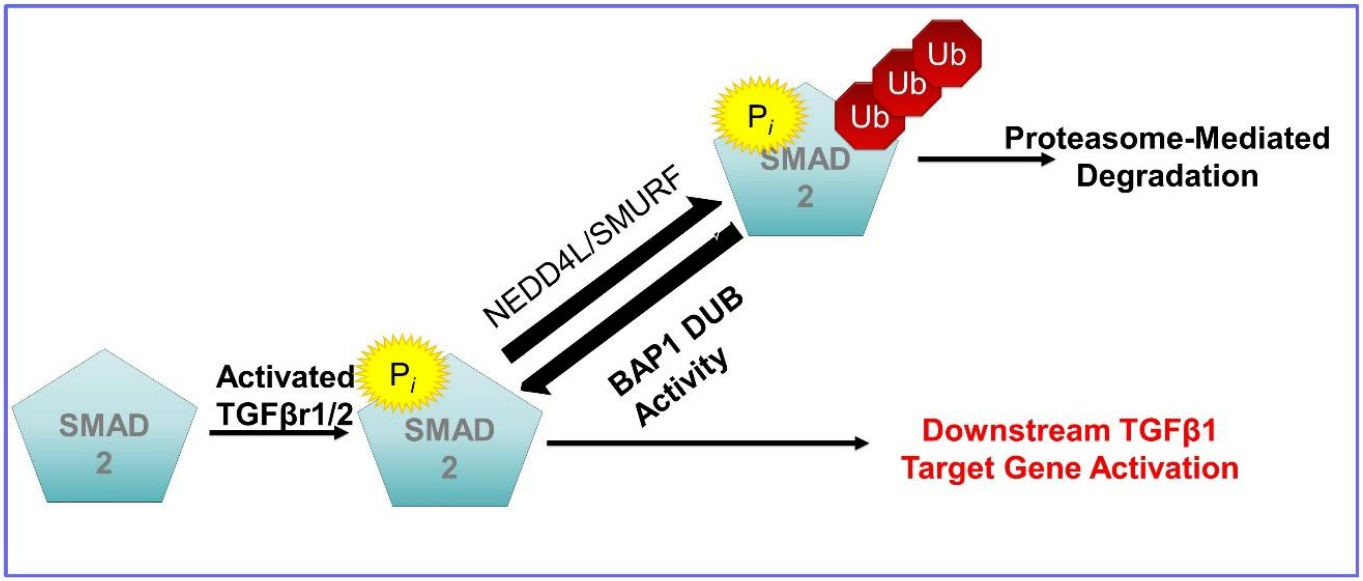

Figure 12. **A Model for BAP1 Regulation of TGFβ Signaling**. SMAD2 is phosphorylated via the activated TGFβr1/2 complex. Activated SMAD2 particles are targeted by NEDD4L or SMAD ubiquitin regulatory factors (SMURFs) for degradation via ubiquitination, which can be downregulated by the de-ubiquitination activity of BAP1. BAP1 thus enhances TGFβ signaling by protecting activated SMAD2 from proteasome-mediated protein degradation.

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