# MOLECULAR REGULATION OF HAIR FOLLICLE STEM AND PROGENITOR CELL HOMEOSTASIS

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# MOLECULAR REGULATION OF HAIR FOLLICLE STEM AND PROGENITOR CELL HOMEOSTASIS

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Maintenance of adult stem cells and their progenitor cells is critical for proper homeostasis of their resident tissues. Defects in tissue stem or progenitor cell maintenance can cause developmental disorders, premature aging, and cancers. Identification of transcription factors regulating these processes have yielded significant insight regarding the mechanisms necessary for tissue homeostasis. The mouse hair follicle provides a dynamic system that is well suited for the study of tissue stem cell and progenitor cell regulation. Utilizing an inducible epithelial knockout system, I investigated the role of the transcription factor *Gata6* in the hair follicle and discovered that *Gata6* is essential for initiation and maintenance of hair follicle growth.

This work found *Gata6* in control of the renewal of rapidly proliferating hair progenitors and hence the extent of production of terminally differentiated lineages. In the absence of *Gata6*, progenitor cells *in vivo* as well as epidermal keratinocytes in culture accumulate DNA damage and are lost to apoptosis. *Gata6* in the hair appears to protect progenitor cells from DNA damage associated with proliferation. This is accomplished, in part, through EDA-receptor signaling adaptor EDARADD and NF-κB pathway activation, known to be important for DNA-damage repair and stress

response in general, and for hair follicle growth in particular. In cultured keratinocytes, *Edaradd* rescued DNA damage, cell survival and proliferation of *Gata6* knockout cells. This *Gata6*-*Edaradd*-NF-κB pathway also activated the minichromosome maintenance factor *Mcm10*, which plays an important role in DNA replication processivity and DNA damage repair in response to replication stress.

My data expands the role of *Gata6*, which has predominantly been recognized as a developmental factor for mesodermal and endodermal tissue lineages, to an ectodermal tissue where it plays a critical role in genome maintenance of progenitor cells. This work adds to other recent work in embryonic stem and neural progenitor cells, suggesting a model whereby developmentally regulated transcription factors protect from DNA damage associated with proliferation at key stages of rapid tissue growth. Our data may also provide a better understanding of why *Gata6* is a frequent target of amplification in cancers and provide potential targets for treating *Gata6* amplified cancers.

# BIOGRAPHICAL SKETCH

Alex Wang was born on November 25, 1987 in Royal Oak, Michigan and grew up in sunny Jacksonville, Florida. Alex attended Stanton College Preparatory School, where he was introduced to the fundamentals of Biology and Chemistry. In 2006, he graduated from Stanton's International Baccalaureate program and entered the University of Florida as a National Merit Scholar.

While at the University of Florida, Alex majored in Biochemistry with a minor in Philosophy. As a freshman, Alex eagerly joined the laboratory of Dr. Connie Mulligan in the Department of Anthropology. Under the guidance of Dr. Mulligan, he utilized genetic variation to examine the origins and directionality of migration of early humans out of Africa. He also conducted research on genetic variation of ancient DNA samples collected from indigenous Puerto Rican Tainos to examine the pre-Columbian colonization of the Caribbean. These experiences motivated Alex to continue pursuing a career in biological research and pursue a PhD in Molecular Biology.

In 2010, Alex entered the Biochemistry, Molecular & Cell Biology program at Cornell University. He was particularly drawn to the excitement and promise of tissue stem cells and joined Dr. Tudorita Tumbar's laboratory in 2011. In the Tumbar lab, Alex has pursued projects investigating regulation of stem cell homeostasis and differentiation. After graduation, Alex will continue his journey in science and will be joining the laboratory of Dr. Bruce Spiegelman at Dana-Farber Cancer Institute will he will be studying regulation of fat cell differentiation and energy homeostasis.

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# CHAPTER 1

# INTRODUCTION TO TISSUE STEM CELL HOMEOSTASIS

## *1.1 Stem cells*

Stem cells have the unique ability to self-renew and differentiate for an extended period of time (Weissman *et al*, 2001). Somatic stem cells, or tissue stem cells, are undifferentiated population of cells found in tissues and organs that are responsible for maintaining and repairing the tissue in which they reside. The stem cells reside in a specialized microenvironment called the niche, which supports the homeostasis of the stem cells and provides signaling cues that regulate the activity of the stem cells (Morrison & Spradling, 2008). The regulation of stem cell activity is regulated through intrinsic mechanisms such as gene expression as well as through extrinsic cues from the niche and the rest of the surrounding environment. Some tissue stem cells such as those of the hair follicle and hematopoietic systems remain notably quiescent for much of their lifetime (**Figure 1.1**). This prolonged quiescence is believed to prevent accumulation of replication associated damage such as DNA damage and telomere shortening, and prevents exhaustion of the stem cell pool that may lead to premature aging (Fuchs, 2009). On the other hand, progenitor cells, intestinal stem cells, and epidermal stem cells display constant turnover. Differentiation of stem cells follows a hierarchical organization, where the stem cells first produce progenitor cells. These progenitor cells, or transit-amplifying cells, are also capable of self-renewal and differentiation but unlike stem cells, they have a limited life span. Particularly for the hair follicle and hematopoietic systems where the

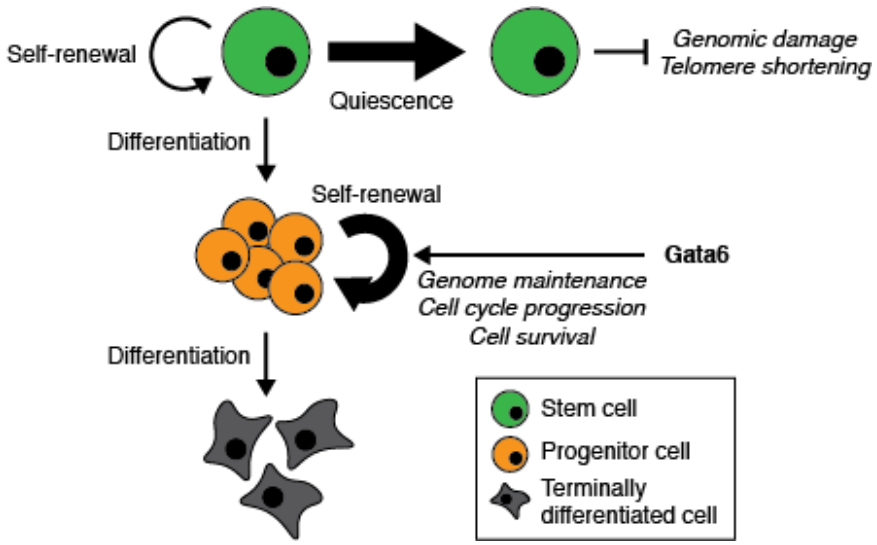



**Figure 1.1** Hierarchical model of stem cell lineage. Tissue stem cells remain quiescent for the majority of their lifetime, thus protecting themselves from replication-associated damage such as DNA damage and telomere shortening. Progenitor cells on the other hand proliferate robustly and are therefore susceptible to replication associated genomic instability.

stem cells are largely quiescent, the progenitor cells must carry the burden of robustly proliferating to provide terminally differentiated cells to maintain homeostasis of the tissue.

As the body's natural source of maintaining and repairing tissues, tissue stem cells are an area of great clinical significance for their potential in regenerative medicine. Additionally, improper stem cell function has been found to give rise to cancer, tissue degeneration, and aging.

Mouse hair follicles provide a valuable model system for studying the dynamic behavior and regulatory mechanisms of stem cells in their native tissue environment. The molecular characterization of hair follicle stem cell (HFSC) quiescence, self-renewal and differentiation has been an area of intense study and a wide variety of signaling pathways and developmental transcription factors have been uncovered for each stage (Lee & Tumbar, 2012). HFSC behavior is also influenced by environmental signaling from the niche and the neighboring non-epithelial cells of the skin. Recently, increasing attention has been drawn to the interaction between the hair follicle and its surrounding environment such as the surrounding dermal fibroblasts and dermal sheath, adipocytes, blood vessels, neurons, immune cells, and arrector pili muscle. Chapter 2 of this dissertation provides a detailed summary of how these environmental components influence the behavior of HFSCs, and how the HFSCs in turn influences and shapes its surrounding environment.

Although HFSCs have been the subject of great scrutiny, relatively little is known regarding the regulation of hair follicle's progenitor cells. Unlike the hair's stem cells, which remain quiescent for much of their lifetime, hair progenitor cells

carry much of the burden of tissue regeneration and must face the consequences of continuous and rapid proliferation. As I will discuss further in Chapter 3, this has significant consequences on the stability of the progenitor cell genome, and in that chapter I discuss my work analyzing the role of the transcription factor *Gata6* in buffering the progenitor cells from replication associated DNA damage.

### *1.2 Developmental roles of transcription factor Gata6*

*Gata6* is a member of the GATA family of zinc finger transcription factors that bind the consensus DNA sequence (T/A)GATA(A/G) (Morrisey *et al*, 1996). All six mammalian family members contain tandem zinc fingers, often referred to as the N-finger and C-finger. While the C-finger primarily binds the GATA motif, the N-finger can bind DNA independently (with a preference for GATC sequences), participate in cooperative binding of proximal palindromic GATA motifs with the C-finger, and engage in protein-protein interactions (Bates *et al*, 2008; Maeda *et al*, 2005). The mammalian GATA transcription factors can be divided into two groups, with *Gata1/2/3* functioning primarily in hematopoietic cells and *Gata4/5/6* primarily in mesodermal and endodermal organs such as the heart and gastrointestinal tissues (Laverriere *et al*, 1994).

*Gata6* is involved in the development and homeostasis of a variety of tissues such as cardiac vascular smooth muscle, intestine, liver, pancreas, ovaries, and lungs (Lentjes *et al*, 2016), and *Gata6* is also the determining factor driving differentiation of the extraembryonic endoderm during early embryogenesis (Morrisey *et al*, 1998). Previously, *Gata6* has been shown to regulate tissue development through a variety of

pathways and mechanisms, and *Gata6*'s multiple roles in controlling cellular proliferation, differentiation and survival are context dependent. In the mouse epiblast, *Gata6* promotes embryonic stem cell survival through upregulation of *Bmp2* (Rong *et al*, 2012). Additionally, during early embryogenesis, *Gata6* promotes differentiation towards the extraembryonic endoderm lineage through upregulation of *HNF4*, FGF/ERK signaling, and repression of pluripotency factors (Morrisey *et al*, 1998; Schrode *et al*, 2014; Wamaitha *et al*, 2015).

In tissue development, *Gata6* regulates development of a variety of endodermal and mesodermal lineages. Intestinal epithelium morphogenesis and differentiation occurs through Notch signaling regulation by *Gata4* and *Gata6* (Walker *et al*, 2014). In lung epithelium development *Gata6* targets alveolar specific markers as well as Wnt signaling receptor *Fzd2* (Yang *et al*, 2002). In contrast to these roles as a differentiation factor, *Gata6* has also been shown to regulate proliferation in several tissues, in both a negative and a positive manner (Xie *et al*, 2015; Walker *et al*, 2014; Carrasco *et al*, 2012; Kamnasaran *et al*, 2007; Perlman *et al*, 1998). Loss of *Gata4* and *Gata6* in the pancreas causes pancreatic agenesis stemming from disruption of pancreatic progenitor cell proliferation (Xuan *et al*, 2012).

While, *Gata6* has been well characterized during development, its role during adult tissue homeostasis has not been well addressed. Peritoneal macrophage proliferation has recently been shown to depend on *Gata6* both during homeostasis and in response to inflammation (Rosas *et al*, 2014). In the adult lung epithelium, loss of *Gata6* was associated with an expansion of bronchial alveolar stem cells and a

concomitant loss of differentiated cells due to disrupted Wnt signaling (Zhang *et al*, 2008).

The importance of *Gata6* in tissue homeostasis is also suggested by the frequency of *Gata6* overexpression and amplifications in various cancers, where it acts as an oncogene involved in proliferative capacity and cell survival (Zheng & Blobel, 2010). Particularly in colorectal cancer and pancreatic adenocarcinoma patients, the expression level of *Gata6* has been found to be predictive of patient survival (Shen *et al*, 2013; Lin *et al*, 2012). *Gata6* is essential for colorectal cancer tumorigenicity and has been shown to affect the proliferation of colorectal cancer cells through regulation of Wnt and Bmp pathways (Whissell *et al*, 2014; Tsuji *et al*, 2014). GATA6 binds a distal enhancer of the growth inhibitory *Bmp4*, thus repressing its expression (Whissell *et al*, 2014). *Gata6* also aberrantly activates Wnt signaling through upregulation of *Lgr5* (Tsuji *et al*, 2014). Similarly, in pancreatic cancers, *Gata6* promotes proliferation in part through repression of the Wnt antagonist *Dkk1* (Zhong *et al*, 2011; Kwei *et al*, 2008). *Gata6* has been found to be a target of recurrent amplification in 21% of esophageal adenocarcinoma samples, which become dependent on *Gata6* for growth and survival (Lin *et al*, 2012). Thus, further study of *Gata6's* role in tissue homeostasis, as will be discussed in Chapter 3, may provide a valuable link to understanding and potentially identifying therapeutic targets for these *Gata6* amplified cancers.

### *1.3 Dissertation Outline*

This dissertation discusses the molecular mechanisms regulating stem and progenitor cell homeostasis and differentiation in the mouse hair follicle. Chapter 2 contains a detailed review of the current research on homeostasis of the HFSC niche. The review focuses on intrinsic and environmental mechanisms that regulate quiescence and activation of the HFSCs, with particular emphasis placed on the interactions between the stem cells, the niche, and the other non-epithelial cells of the skin that make up the microenvironment and contribute to hair follicle homeostasis (Wang *et al*, 2015).

Chapter 3 analyzes the importance of the transcription factor *Gata6* on the self-renewal of hair follicle progenitor cells. Loss of *Gata6* in the skin epithelium causes proliferation associated DNA damage and apoptosis specifically in the hair follicle's progenitor cells. Our data suggest that *Gata6* is critical for genome maintenance of progenitor cells during proliferation, in part through transcriptional activation of the ectodysplasin A receptor associated death domain protein *Edaradd*.

Chapter 4 provides a summary of the main conclusions described in the previous chapter and implications of this work on the broader understanding of stem cell regulation. This chapter also provides an outlook on future directions suggested by these findings.

REFERENCES

Bates DL, Chen Y, Kim G, Guo L & Chen L (2008) Crystal Structures of Multiple GATA Zinc Fingers Bound to DNA Reveal New Insights into DNA Recognition and Self-Association by GATA. *J Mol Biol* **381:** 1292–1306

Carrasco M, Delgado I, Soria B, Martín F & Rojas A (2012) GATA4 and GATA6 control mouse pancreas organogenesis. *J. Clin. Invest.* **122:** 3504–3515

Fuchs E (2009) The tortoise and the hair: slow-cycling cells in the stem cell race. *Cell* **137:** 811–819

Kamnasaran D, Qian B, Hawkins C, Stanford WL & Guha A (2007) GATA6 is an astrocytoma tumor suppressor gene identified by gene trapping of mouse glioma model. *Proc Natl Acad Sci USA* **104:** 8053–8058

Kwei KA, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi Y-L, Chatterjee S, Karikari CA, Salari K, Wang P, Hernandez-Boussard T, Swarnalata G, van de Rijn M, Maitra A & Pollack JR (2008) Genomic Profiling Identifies GATA6 as a Candidate Oncogene Amplified in Pancreatobiliary Cancer. *PLoS Genet* **4:** e1000081

Laverriere AC, MacNeill C, Mueller C, Poelmann RE, Burch JB & Evans T (1994) GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J Biol Chem* **269:** 23177–23184

Lee J & Tumbar T (2012) Hairy tale of signaling in hair follicle development and cycling. *Semin. Cell Dev. Biol.* **23:** 906–916

Lentjes MHFM, Niessen HEC, Akiyama Y, de Bruïne AP, Melotte V & van Engeland

M (2016) The emerging role of GATA transcription factors in development and disease. *Expert Rev Mol Med* **18:** e3

Lin L, Bass AJ, Lockwood WW, Wang Z, Silvers AL, Thomas DG, Chang AC, Lin J, Orringer MB, Li W, Glover TW, Giordano TJ, Lam WL, Meyerson M & Beer DG (2012) Activation of GATA binding protein 6 (GATA6) sustains oncogenic lineage-survival in esophageal adenocarcinoma. *Proc Natl Acad Sci USA* **109:** 4251–4256

Maeda M, Ohashi K & Ohashi-Kobayashi A (2005) Further extension of mammalian GATA-6. *Dev. Growth Differ.* **47:** 591–600

Morrisey EE, Ip HS, Lu MM & Parmacek MS (1996) GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev. Biol.* **177:** 309–322

Morrisey EE, Tang Z, Sigrist K, Lu MM, Jiang F, Ip HS & Parmacek MS (1998) GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes & Development* **12:** 3579–3590

Morrison SJ & Spradling AC (2008) Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132:** 598–611

Perlman H, Suzuki E, Simonson M, Smith RC & Walsh K (1998) GATA-6 induces p21(Cip1) expression and G1 cell cycle arrest. *J Biol Chem* **273:** 13713–13718

Rong L, Liu J, Qi Y, Graham AM, Parmacek MS & Li S (2012) GATA-6 promotes cell survival by up-regulating BMP-2 expression during embryonic stem cell differentiation. *Mol Biol Cell* **23:** 3754–3763

Rosas M, Davies LC, Giles PJ, Liao C-T, Kharfan B, Stone TC, O'Donnell VB, Fraser

DJ, Jones SA & Taylor PR (2014) The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal. *Science* **344:** 645–648

Schrode N, Saiz N, Di Talia S & Hadjantonakis A-K (2014) GATA6 levels modulate primitive endoderm cell fate choice and timing in the mouse blastocyst. *Dev Cell* **29:** 454–467

Shen F, Li J, Cai W, Zhu G, Gu W, Jia L & Xu B (2013) GATA6 predicts prognosis and hepatic metastasis of colorectal cancer. *Oncol. Rep.* **30:** 1355–1361

Tsuji S, Kawasaki Y, Furukawa S, Taniue K, Hayashi T, Okuno M, Hiyoshi M, Kitayama J & Akiyama T (2014) The miR-363-GATA6-Lgr5 pathway is critical for colorectal tumourigenesis. *Nat Commun* **5:** 3150

Walker EM, Thompson CA & Battle MA (2014) GATA4 and GATA6 regulate intestinal epithelial cytodifferentiation during development. *Dev. Biol.* **392:** 283–294

Wamaitha SE, del Valle I, Cho LTY, Wei Y, Fogarty NME, Blakeley P, Sherwood RI, Ji H & Niakan KK (2015) Gata6 potently initiates reprograming of pluripotent and differentiated cells to extraembryonic endoderm stem cells. *Genes & Development* **29:** 1239–1255

Wang AB, Jain P & Tumbar T (2015) The Hair Follicle Stem Cell Niche: The Bulge and Its Environment. In *Tissue-Specific Stem Cell Niche* pp 1–26. Cham: Springer International Publishing

Weissman IL, Anderson DJ & Gage F (2001) Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu. Rev. Cell Dev. Biol.* **17:** 387–403

Whissell G, Montagni E, Martinelli P, Hernando-Momblona X, Sevillano M, Jung P, Cortina C, Calon A, Abuli A, Castells A, Castellvi-Bel S, Nacht AS, Sancho E, Stephan-Otto Attolini C, Vicent GP, Real FX & Batlle E (2014) The transcription factor GATA6 enables self-renewal of colon adenoma stem cells by repressing BMP gene expression. *Nat Cell Biol* **16:** 695–707

Xie Y, Jin Y, Merenick BL, Ding M, Fetalvero KM, Wagner RJ, Mai A, Gleim S, Tucker DF, Birnbaum MJ, Ballif BA, Luciano AK, Sessa WC, Rzucidlo EM, Powell RJ, Hou L, Zhao H, Hwa J, Yu J & Martin KA (2015) Phosphorylation of GATA-6 is required for vascular smooth muscle cell differentiation after mTORC1 inhibition. *Sci Signal* **8:** ra44–ra44

Xuan S, Borok MJ, Decker KJ, Battle MA, Duncan SA, Hale MA, Macdonald RJ & Sussel L (2012) Pancreas-specific deletion of mouse Gata4 and Gata6 causes pancreatic agenesis. *J. Clin. Invest.* **122:** 3516–3528

Yang H, Lu MM, Zhang L, Whitsett JA & Morrisey EE (2002) GATA6 regulates differentiation of distal lung epithelium. *Development* **129:** 2233–2246

Zhang Y, Goss AM, Cohen ED, Kadzik R, Lepore JJ, Muthukumaraswamy K, Yang J, DeMayo FJ, Whitsett JA, Parmacek MS & Morrisey EE (2008) A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. *Nat Genet* **40:** 862–870

Zheng R & Blobel GA (2010) GATA Transcription Factors and Cancer. *Genes Cancer* **1:** 1178–1188

Zhong Y, Wang Z, Fu B, Pan F, Yachida S, Dhara M, Albesiano E, Li L, Naito Y, Vilardell F, Cummings C, Martinelli P, Li A, Yonescu R, Ma Q, Griffin CA, Real

FX & Iacobuzio-Donahue CA (2011) GATA6 Activates Wnt Signaling in Pancreatic Cancer by Negatively Regulating the Wnt Antagonist Dickkopf-1. *PLoS ONE* **6:** e22129

CHAPTER 2

# THE HAIR FOLLICLE STEM CELL NICHE: THE BULGE AND ITS ENVIRONMENT [1]

## *2.1 Summary*

The hair follicle (HF) is a dynamic miniorgan that has become one of the best understood models of tissue stem cell (SC) behavior. Distinct temporal orchestration of HF SC self-renewal, differentiation, and quiescence makes the HF an ideal system for studying regulation in a SC niche during normal homeostasis of a tissue. The HF bulge structure acts as the SCs maintenance niche, and houses a heterogeneous collection of SCs that promote HF growth and contribute to epidermal wound repair. Bulge-neighboring cells contribute to the niche environment and are important for regulating HFSC activation, quiescence and differentiation. This review will explore the HFSC behavior within their niche, and the mechanisms that contribute to the homeostasis and maintenance of HFSCs in adult skin.

## *2.2 Introduction*

The mouse hair follicle (HF) has emerged as a powerful system for studying the behavior of stem cells (SCs) within their native tissue context. A major focus of the field has been to understand the regulatory mechanisms underlying self-renewal,

[1] This chapter has been published and is reprinted here with permission: Wang, A.B., Jain, P., and Tumbar, T (2015). "The Hair Follicle Stem Cell Niche: The Bulge and Its Environment." In Turksen K. (Ed.), *Tissue-Specific Stem Cell Niche* (pp. 1-26). Springer. Author contributions: P.J. contributed to writing in Section 2.9 and Figure 2.3; A.B.W. and T.T. wrote the paper.

differentiation, and quiescence. In the HF, this effort has greatly benefitted from the fact that its SCs and progeny with different degrees of differentiation are spatially and temporally restricted.

The HF is composed of distinct compartments of SCs, progenitor cells, and terminally differentiated cells. The hair follicle stem cells (HFSCs) are located in a region of the follicle called the bulge. This anatomical feature is an outcropping of the outer root sheath (ORS), the outermost layer surrounding the HF, and is located below the sebum producing structure, known as the sebaceous gland (SG). The bulge behaves as a "storage" niche that contains a large population of clustered SCs. SCs within the bulge may remain in the niche and self-renew for extensive periods of time, or they may be expelled from the bulge to become early progenitor cells in the hair germ at the base of the hair. The hair germ expands by divisions to form the matrix cells, which constitute bona fide, mature progenitor cells that subsequently terminally differentiate to form concentric layers of post-mitotic cells known as the inner root sheath (IRS) and the hair shaft in the center.

The HF cycles through stages of growth (anagen), regression (catagen), and rest (telogen), which occur synchronously across the back of the mouse [1] (**Figure 2.1**). In anagen, the bulge SCs divide on average three times [2] and self-renew while remaining confined to the bulge [3]. In parallel, matrix progenitor cells proliferate and differentiate to fuel the growing hair shaft. Then, during catagen, the follicle below the bulge undergoes extensive apoptosis of the progenitor and differentiated cells, leaving a quiescent bulge to rest during telogen [4-6]. The cycling of the HF depends on signals originating from the dermal papilla (DP), a cluster of mesenchymal cells at the

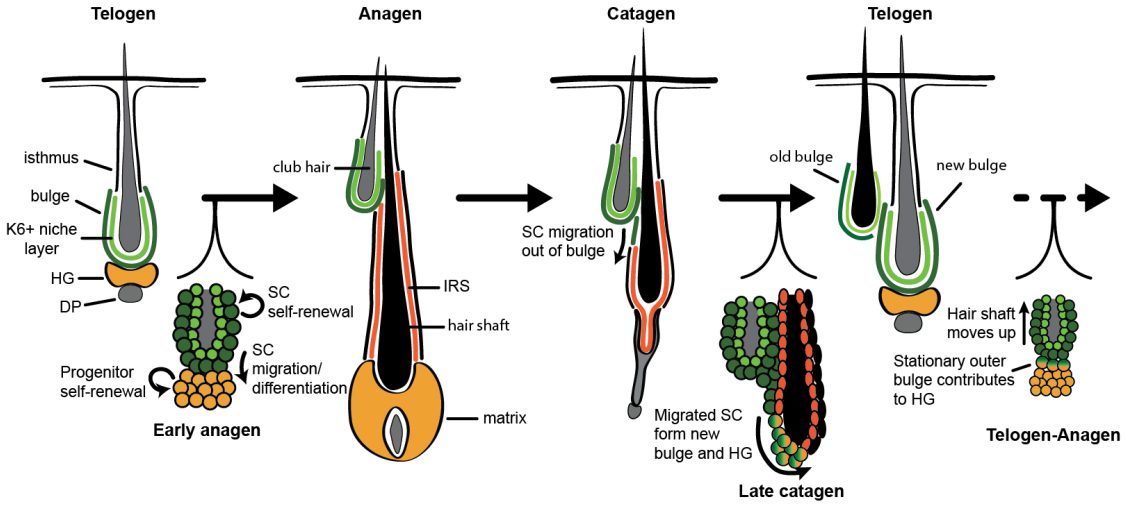



**Figure 2.1** During the hair cycle, HFs undergo synchronous stages of rest (telogen), growth (anagen), and regression (catagen). Stem cell activation occurs during the transitions between telogen-anagen (self-renewal) and catagen-telogen (differentiation) (highlighted in the offshoots between stages). During the transition from telogen to anagen, progenitor cells of the hair germ proliferate to form the matrix and bulge SCs either migrate into the hair germ to differentiate or remain in the bulge to self-renew. During the transition from catagen to telogen, bulge SCs first migrate out of the bulge into the ORS then as catagen ends, the migrated SCs form a new bulge around the hair shaft and a new hair germ beneath the new bulge. During telogen and into early anagen, the hair shaft continues to be pushed upwards, leaving some outer bulge cells to collapse around its former position and contribute more bulge cells to the new hair germ.

base of the HF, and from the surrounding environment [7-9]. This includes dermal fibroblasts, blood vessels, nerves, fat, immune cells, and the arrector pili muscle, which are non-epithelial cells that surround the HF and influence HF activation and growth. In this review, we will highlight the mechanisms regulating the behavior of HFSCs as well as their interaction with various niche components during the hair cycle, both within the HF as well as non-epithelial components near the follicle. Additionally, at the end of the review we will discuss mechanisms utilized by HFSCs for quiescence, long-term maintenance, and cancer prevention.

### *2.3 The bulge as the epithelial hair follicle stem cell niche*

The bulge was originally posited to be the HFSC niche based on the slow-cycling properties of cells located in the outermost layer, as determined by label retention in nucleotide analog pulse-chase experiments [10]. In these experiments, the mouse is pulsed with repeated doses of labeled nucleotides such as tritiated thymidine ($^{3}$HTdR) or bromodeoxyuridine (BrdU) to label all of the proliferative cells. During the chase period (4-10 weeks), as expected, the short-lived but rapidly dividing progenitor cells of the matrix and the lower ORS dilute the label with each cell division until it is eventually lost. On the other hand, as expected of tissue SCs, bulge cells rarely divide and therefore remain as label retaining cells (LRCs). Dissection and transplantation of lacZ or GFP labeled HF bulges or derivatives from single cells, as well as characterization of growth potential in cell culture, provided functional evidence that the outer bulge layer contains the SCs of the HF and could differentiate and contribute to all lineages of the HF [11, 12].

The pulse-chase labeling method to identify LRCs was later upgraded with the generation of a transgenic mouse with a doxycycline inducible histone H2B-GFP labeling system, allowing live LRCs to be purified by fluorescence activated cell sorting [13]. Alternatively, the outer bulge cells can also be purified using cell surface markers CD34 and α6-integrin [14] or transgenic labeling with the bulge specific keratin 15 (K15) promoter driving Cre recombinase expression, although K15 expression has also been reported in the hair germ during telogen [15, 16]. Sorting and transcriptional profiling of the LRCs have revealed a variety of transcription factors and signaling pathways that have greatly contributed to our understanding of how HFSC behavior is regulated (discussed in subsequent sections) [9, 17].

In addition to the bulge outer layer that contains the SCs, the bulge also contains an inner keratin 6 (K6)+ niche layer that serves to anchor the old hair (club hair) and promote bulge SC quiescence [18]. K6 is also expressed in the terminally differentiated companion layer between the ORS and IRS during anagen, but unlike this layer, the K6+ inner bulge layer shows expression of HFSC markers such as *Sox9*, *Lhx2*, *Tcf3*, and *NFATc1*. Unlike the other cells in the bulge, the K6+ inner layer cells make extensive adhesive contacts with the club hair and are post-mitotic [18]. The inner bulge layer constitutes the epithelial component of the HFSC niche and is known to act in synergy with the perifollicular non-epithelial components (discussed further in Section 2.8) to establish a niche environment that regulates HFSC quiescence and activation.

### *2.4 Quiescence within the bulge*

As with some, although not all tissue SC populations, bulge SCs remain quiescent for the majority of their lifetime [19, 20]. Quiescence is maintained both by internal programming of the SCs as well as by external signaling. The major molecular driver of bulge quiescence is BMP signaling. During telogen, the DP, hair germ, and surrounding dermis express *Bmp4* [21], and the bulge inner layer expresses *Bmp6* [18]. Conditional deletion of *Bmpr1a* in the bulge causes the quiescent SCs to precociously activate and continuously proliferate without differentiation to form tumor-like cysts [22]. Conversely, introduction of ectopic Bmp by injection of BMP4-soaked beads into the skin is sufficient to prevent anagen initiation and to maintain quiescence [21]. BMP signaling has been shown to maintain quiescence in the HFSCs in part through the upregulation of the transcription factor *NFATc1*. *NFATc1* is expressed in the quiescent bulge cells throughout the hair cycle and maintains quiescence at least in part by repressing the cyclin-dependent kinase CDK4 [23].

As mentioned previously, the bulge contains an inner layer of K6+ cells that promotes quiescence in the surrounding outer SC layer. Loss of the K6+ layer by hair plucking or ablation with iDTR in *Sox9-CreER* mice induces the surrounding CD34+ bulge cells to proliferate and initiate anagen [18, 24]. In both cases, not only the inner K6 layer but also some of the outer, SC containing bulge layer is lost, raising questions about data interpretation attributing loss of SC quiescence solely to damage of the inner layer. Supporting the model in which the K6+ inner layer is required for SC quiescence, loss of cells from the outer layer alone via *K15-Cre*, led to less robust induction of SC proliferation. The specific mechanisms required in the communication between the outer SC layer of the bulge and the inner K6+ layer remain to be

established, although it has been suggested that BMP secretion from the inner layer might play a central role [18].

Quiescence is an essential strategy for long-term SC survival. Over-proliferation of the bulge poses the risk of premature hair loss due to exhaustion of the SC pool [25-27]. To promote quiescence, HFSCs express high levels of cyclin-dependent kinase inhibitors (CDKIs) such as *p21*, *p27*, *p57*, and *p15* [28, 29]. While the expression of multiple CDKIs likely provides some redundancy, *p21* has been implicated in ensuring that activated HFSCs promptly exit the cell cycle at early catagen after a defined number of divisions. Specifically, the *p21* knockout HFSCs show on average one to two additional cell divisions prior to entering quiescence potentially due to increased apoptosis and available space in the SC niche [29]. Because *p21* was upregulated in the *Runx1* KO, which showed prolonged telogen [30, 31], it was possible that *p21* might be responsible for blocking HF proliferation in this genetic background. Surprisingly, in a *p21*-null genetic background, *Runx1* knockout induced quiescence was not overcome, but instead it was even further and excessively prolonged [29]. This raised the puzzling question as to how inhibiting a cell cycle inhibitor (*p21*) resulted in enhanced quiescence, the opposite effect from the expected. The explanation might reside in the specific upregulation of other CDKIs, in particular p15, which occurs only in the double *p21* and *Runx1* knockout. *P21* has been previously shown to have a dual role as both a CDKI and transcriptional repressor in skin epithelial cells [32], and more recently *p27* was shown to work in a similar manner in a different system [33]. Intriguingly, it appears that *p21* and *Runx1* synergistically repress the *p15* promoter at the transcriptional level [29]. This suggests

a feedback mechanism in HFSCs that reinforces quiescence by regulating the levels of CDKIs within SCs via the dual function of some CDKIs as cyclin dependent kinase inhibitors and transcriptional repressors [29]. This CDKI mechanism as well as quiescence promoting signaling from the DP and inner bulge layer highlights the variety of methods used to keep HFSCs quiescent. Beyond ensuring that enough HFSCs are maintained to sustain hair growth, quiescence also plays an important role in tumor suppression, which we will discuss later in the review (see section 2.9).

### *2.5 Activation of HFSCs*

Bulge activation occurs during two temporally distinct stages: activation for self-renewal occurs during early anagen, whereas activation for differentiation primarily occurs during catagen/telogen [34, 3]. At the beginning of anagen, activation of proliferation occurs in two steps in two HF compartments, first in the hair germ, which is fated to differentiate, and second in the bulge, which self-renews.

The hair germ is a structure located at the base of the telogen HF between the bulge and the DP. The hair germ originates from SCs that migrated out of the bulge into the ORS during early catagen [35, 34, 18]. These cells escape apoptosis during catagen and remain as a cluster of cells beneath the bulge in telogen. These cells are thought of as differentiation "primed" SCs or early progenitor cells, also referred to as activated SCs. When removed from their native tissue environment and plated in cell culture, hair germ cells show a more rapid initial colony formation and short-term expansion than bulge cells, followed by exhaustion and inability to survive multiple passages [34]. Rapid growth and short-term survival are well-established

characteristics that define progenitor cells. As of yet, the mechanisms governing this transition from HFSCs to hair germ progenitor cells are not well understood. However, our lab has recently found that the quiescent HFSCs that migrate out of the bulge express high levels of the transcription factor *Runx1* as they lose contact with the inner bulge layer [36]. In this study, transgenic overexpression of *Runx1* was sufficient to induce catagen and to drive the transition of HFSCs to the progenitor fate, as shown by gene expression profiling and functional studies.

While an initial hair germ forms from bulge cell migration at catagen [35, 34, 18], bulge cells continue to contribute additional cells to the pre-existing hair germ during telogen and very early anagen [3, 37]. This was demonstrated by single bulge cell lineage tracing from our laboratory, in which tracking the distribution of LacZ+ cells within the bulge suggested that during telogen the entire bulge outer layer is shifted downwards with respect to the hair shaft adding new cells to the pre-existing hair germ [3]. The most likely explanation is that the club hair continues to move upwards, dragging the inner bulge layer with it and leaving cells from the outer bulge layer to collapse underneath, thus enlarging the hair germ. Bulge cells that lose contact with the inner layer gain *Runx1* expression and as a consequence lose CD34, thus becoming new hair germ cells during telogen/early anagen [3, 36].

Under normal homeostasis, the hair germ is fated to divide in anagen and expand into the matrix, which in turn will terminally differentiate [38]. However, upon injury to the bulge, the hair germ cells are capable of returning to the bulge to replace the lost SCs [35, 38]. This suggests that the hair germ is not fully committed to a progenitor fate, and is consistent with the reversible effects of *Runx1* expression on

turning the bulge cells to a hair germ phenotype [36]. In accordance with its position directly above the DP, the hair germ is first to receive the activating WNT and FGF signals at the start of anagen and begin proliferation [34]. There must also be a special intrinsic state of these cells that allows them to readily respond to activation signals, as suggested by their rapid growth in cell culture. This "activated" state is likely induced by *Runx1* through its many target genes implicated in cell size growth and active metabolism, and is confirmed by the rapid growth of keratinocytes with elevated *Runx1* levels and by delayed activation of hair germ cell proliferation in the *Runx1* epithelial knockout mouse [30, 31, 39, 36].

After the hair germ became activated to divide and form the matrix, a second wave of activation triggers proliferation of the HFSCs in their bulge location. Based on the physical separation of the bulge from the activating signals of the DP and the delayed proliferation following hair germ expansion at anagen initiation, it has been proposed that the proliferating matrix cells actually provide the activating signal for HFSCs rather than the DP. Unlike the hair germ, the bulge does not show activation of WNT and TGF-β pathways, whose signals originate from the DP, but instead the bulge depends on sonic hedgehog (SHH) signaling originating from the matrix [40]. In order to overcome the distance between the bulge and the matrix, the bulge increases its sensitivity to SHH by upregulating hedgehog coreceptors such as *Gas1* [40]. Self-renewal of HFSCs occurs in parallel with the differentiation of the matrix cells; the latter is pushed downwards as the HF bulb grows into the dermis, thus distancing the SHH-activating matrix signals from the bulge SCs. After a few divisions the bulge outer layer cells become extremely crowded, likely triggering contact inhibition

signals via the Hippo/Yap pathway and *p21* [41, 29] to synergize with fading activation signals to reinstate bulge-cell quiescence during mid-anagen.

Bulge HFSCs can also be precociously activated in response to wounding. Upon transplantation or skin wounding, bulge cells display mutipotency and will contribute to neighboring epithelial populations such as the epidermis and the SG [42, 11, 13]. Precociously activated HFSCs migrate out of the bulge and contribute to epidermal wound healing by acting as short-lived progenitors rather than serving as long-term epidermal SCs [43]. Similarly, the SG is maintained independently from the bulge unless disturbed by wounding [44]. Instead, SCs in the isthmus above the bulge may normally contribute to both the epidermis and sebaceous gland during homeostasis [45, 46].

As can be seen, activation of bulge SCs is very tightly controlled, only transiently activating during narrow windows at the beginning of anagen, catagen, and in response to wounding. Interestingly, the bulge niche exclusively houses quiescent and self-renewing SCs, while cells are first exported out of the bulge before differentiation occurs. Presumably signaling within the bulge niche inhibits differentiation, and further investigation into the mechanisms of how the bulge maintains this delineation will be of great interest for the future.

### *2.6 Heterogeneity within the bulge*

Among HFSCs in the bulge there is heterogeneity. Although all HFSCs have the potential to self-renew or differentiate, the two fates do not actually occur at equal frequencies. Single-bulge cell lineage tracing reveals that during early anagen, most

HFSCs remain in the bulge to self-renew through symmetric divisions, as indicated by the orientation of doublets parallel to the basement membrane during early chases, or remain quiescent. On the other hand, only a minority (14%) of the telogen bulge is exported into the pre-existing hair germ to differentiate [2, 3]. Live-cell imaging has confirmed the orientation of cell divisions relative to the basement membrane and further revealed that fate selection of the anagen bulge depends on location of the HFSC within the vertical axis of the bulge [38]. With this method, Rompolas *et al*. genetically labeled single bulge cells with *K19-CreER;Rosa-stop-tdTomato* mice, then lineage traced the fate of the labeled cell *in vivo* through the hair cycle. In general, HFSCs in the upper half of the telogen bulge either remain quiescent or undergo self-renewal and remain in the bulge during anagen and the subsequent telogen. On the other hand, HFSCs in the lower half of the telogen bulge are more likely to exit the bulge into the ORS in late anagen, and become hair germ cells in the subsequent catagen. Labeled hair germ cells exclusively contribute to the differentiated lineages in the subsequent anagen. While the molecular differences between the upper and lower bulge have yet to be characterized, quiescence-related *NFATc1* expression is more prominent in the upper bulge while the gene associated with more actively cycling cells, *Lgr5* (see below), is preferentially detected in the lower bulge. The relationship between location and HFSC fate choice is likely a product of gradients of signaling molecules that pattern the tissue prior to SC activation.

In addition to differences in upper and lower bulge SCs, there is also additional heterogeneity of the bulge cells due to exact relationship to the basement membrane. There are two populations of basal bulge cells that both express CD34 and have label

retention ability, but one of them is more inner relative to the basement membrane, the so-called "suprabasal" SCs, which display lower levels of α6-integrin [28]. FACS isolated samples of the two populations display similar colony formation capacity in cell culture and share similar gene expression profiles. However, *in vivo,* the suprabasal cells are less proliferative based on BrdU labeling and show a corresponding upregulation of the growth inhibitory factors *Fgf18* and *Bmp6*. The significance of this heterogeneity remains unknown.

The basal layer of the lower bulge also contains LGR5+ SCs [47]. LGR5+ bulge cells are biased towards the lateral side opposite the old bulge [47], which shows greater proliferation in anagen [2, 37]. Furthermore, separating bulge cells based on their number of divisions as indicated by H2B-GFP levels showed high *Lgr5* mRNA expression in the more actively cycling cells and low expression in the cells that divided 0-1 times [3]. Thus, LGR5+ cells represent the more actively cycling population, and consistent with this conclusion lineage tracing places them mostly in the proliferative hair germ and the anagen ORS [47]. Lineage tracing also shows that the LGR5+/CD34+ cells in the upper ORS, which are SCs that escaped the old bulge during late anagen/early catagen, are capable of surviving long-term through the hair cycle and contribute to the new bulge SCs, K6+ inner bulge layer, and hair germ [47, 18]. The LGR5+ cells largely overlap with the RUNX1+ cell population, which have been tracked by lineage tracing in a similar manner with LGR5 [36].

The functional significance of *Lgr5* negative cells, which are the more quiescent LRCs that reside in the old bulge or remain on one bulge side after the old bulge is destroyed, remains unclear. Although, it has been proposed that they act as a

reserve population of SCs [18], selective lineage tracing or killing of the most quiescent LRCs in the bulge has yet to be performed. Some of these LRCs from the old bulge seem to migrate laterally along the basement membrane during catagen, also contributing cells to the new bulge, without having to travel downwards through the upper ORS [37].

In addition to housing the epithelial HFSCs, the bulge is also the niche for the neural crest derived melanocyte SC (MSC) population [48]. MSC activation and differentiation follow a similar spatial-temporal pattern as the epithelial HFSCs [49]. Epithelial targeted mutations that block hair cycle progression also block MSC activation. At the beginning of anagen, MSCs contribute cells to the matrix. These cells proliferate in synchrony with the matrix epithelial cells and generate mature melanocytes in the inner cortex of the HF. These cells produce a pigment called melanin and transfer this pigment to the epithelial matrix cells differentiating into the hair shaft. During catagen, the progenitor and mature melanocytes undergo apoptosis along with the epithelial cells of the matrix. In the bulge, MSCs can be distinguished from epithelial HFSCs by their expression of dopachrome tautomerase (DCT) and tyrosine kinase receptor (KIT), which are specific to the melanocyte lineage [50].

The coordination between MSC activity and hair growth is achieved through activating Wnt and inhibitory TGF-β signaling, and crosstalk between HFSCs and MSCs [51]. In the bulge, TGF-β signaling from HFSCs is critical for maintaining MSC quiescence [50]. Further highlighting the importance of HFSCs on MSCs, conditional ablation of the transcription factor *NFIB* in HFSCs promotes aberrant MSC differentiation, implicating *NFIB*'s downstream target endothelins as important

messengers between the SC populations [51]. Additionally, in anagen, Wnt signaling originating from the hair germ initiates MSC proliferation [52]. While HFSCs are critical for the maintenance of its neighboring MSCs, the converse influence of MSCs on HFSCs seems less critical, as MSCs are lost during aging with minimal effects on hair growth which continues to be independently driven from the epithelial compartment (see also Section 2.9 on aging).

The heterogeneity present in the epithelial bulge population is likely a reflection of localized differences in signaling environments determined by relative proximity to major signaling sources within the hair such as the bulge inner layer as well as from the DP and other non-epithelial neighbors of the bulge. The advent of advanced techniques for studying individual cells, such as single cell sequencing and live imaging is likely to reveal further heterogeneity in the behavior of bulge cells. Furthermore, examination of the heterogeneity in bulge behavior will be essential to our understanding of the mechanisms that dictate HFSC fate selection.

### *2.7 Progenitor cells of the hair follicle*

Although HFSCs have been the subject of great scrutiny, relatively little is known regarding the regulation of hair follicle's progenitor cells. The quiescent early progenitor cells of the hair germ are molecularly distinct from the bulge stem cells and the mature proliferative progenitor cells of the anagen hair matrix [34]. In culture, hair germ cells behave as expected for typical progenitor cells: rapid colony formation with inability to survive long-term over multiple passages [34]. However, *in vivo*, hair germ

cells are capable of dedifferentiating back to the stem cell state following loss of the bulge stem cells due to injury [35, 38] or in response to *Runx1* expression levels [36].

Hair germ cells are induced to proliferate upon signaling from the DP at the start of anagen. At the telogen-anagen transition, the DP upregulates the growth stimulatory *Fgf7*, *Fgf10*, and *Wnt10a/b*, while also downregulating the growth inhibitory *Bmp4* [34, 53, 22]. Upon receiving these signals, the hair germ begins rapidly expanding to form the hair matrix. Following initiation, the matrix asymmetrically expresses Sonic Hedgehog (*Shh*) on one side of the follicle to facilitate crosstalk with the progenitor cells and the DP [54]. SHH signaling stimulates expression of *Fgf7* and other growth promoting factors in the DP, thus maintaining progenitor proliferation.

In total, matrix progenitor cells are able to differentiate into seven morphologically and molecularly distinct lineages, which make up the hair shaft its concentric surrounding layers. The hair shaft lies at the center of the hair follicle and consists of the pigmented medulla, cortex, and cuticle. The medulla expresses the trichohyalin protein AE15, while the cortex and cuticle express hair shaft specific kertain AE13 as well as nuclear Wnt coactivators LEF1 and β-catenin [55, 56]. Surrounding the hair shaft are the three layers that compose the IRS: the cuticle of the IRS, Huxley's layer, and Henle's layer [1]. All three layers of the IRS express AE15, while the cuticle and Huxley's layer also express the transcription factor GATA3 [57]. Finally, a keratin 6 expressing companion layer is located between the IRS and outer root sheath.

Lineage tracing during telogen and anagen has shown that while early progenitor cells of the hair germ are multipotent, once the matrix has formed in anagen, the mature progenitors become lineage restricted to either IRS or hair shaft differentiation fates depending on their location within the matrix [58-60]. The lineage specification of the progenitor cells is organized based on the position of the cell along the DP, which is now enveloped by the matrix in anagen. Each position along the length of the DP feeds into a different terminally differentiated layer of the hair follicle, with IRS committed cells emanating from the base of the matrix and hair shaft committed cells coming from the top of the matrix [58, 59]. Additionally, proximity to the DP correlates with the cells proliferative capacity. Cells in the inner matrix adjacent to the DP are continuously self-renewing progenitor cells, while the cells more distal to the DP have limited proliferative capacity until they become terminally differentiated and contribute to the IRS or hair shaft [58]. As the matrix cells reach the IRS and hair shaft, they begin expressing Bmp ligands, which allow them to cease proliferation and begin their terminal differentiation programming [61].

Terminal differentiation of matrix progenitor cells has also been fairly well characterized, with a variety of transcription factors identified for both differentiation of the IRS (CUTL1, GATA3, and FOXN1) and hair shaft (DLX3, FOXN1, MSX2, HOXC13, and LEF1) [62, 57, 63-67, 56]. However, unlike this final step and the earlier regulation of the bulge stem cells, regulation of hair follicle progenitor cell activation and maintenance during homeostasis remains poorly understood. We have recently found evidence suggesting that the rapid and sustained proliferation of matrix cells has significant consequences to the stability of the progenitor cell genome [68].

We found that the developmental transcription factor *Gata6* is essential for the maintenance of progenitor cell renewal, possibly through upregulation of DNA replication and repair components to buffer the progenitor cells from replication associated DNA damage.

### *2.8 Interactions with the non-epithelial environment*

An emerging area of investigation is how the HF stem cells interact with its surrounding environment. A variety of non-epithelial components are found neighboring the HF and contribute to the niche (**Figure 2.2**). As previously mentioned, the DP is ensconced at the base of the HF and serves as a major signaling hub for the follicle. In addition, the entire HF is surrounded by a dermal sheath with a permanent dermal SC population residing at the base of the hair, a ring of blood vessels and nerves that wrap around the isthmus directly above the bulge, the arrector pili muscle attaches directly to the bulge, and adipocytes and immune cells are found in the surrounding dermis. These neighbors display a range of influences on the bulge cell behavior and on hair growth. The DP, adipocytes, and blood vessels are required for proper HF activation because they provide the follicle with key activation cues or nutrients. On the other hand, neighboring neurons and macrophages appear to allow the HF to adjust HFSC activation in response to the environmental conditions such as wounding.

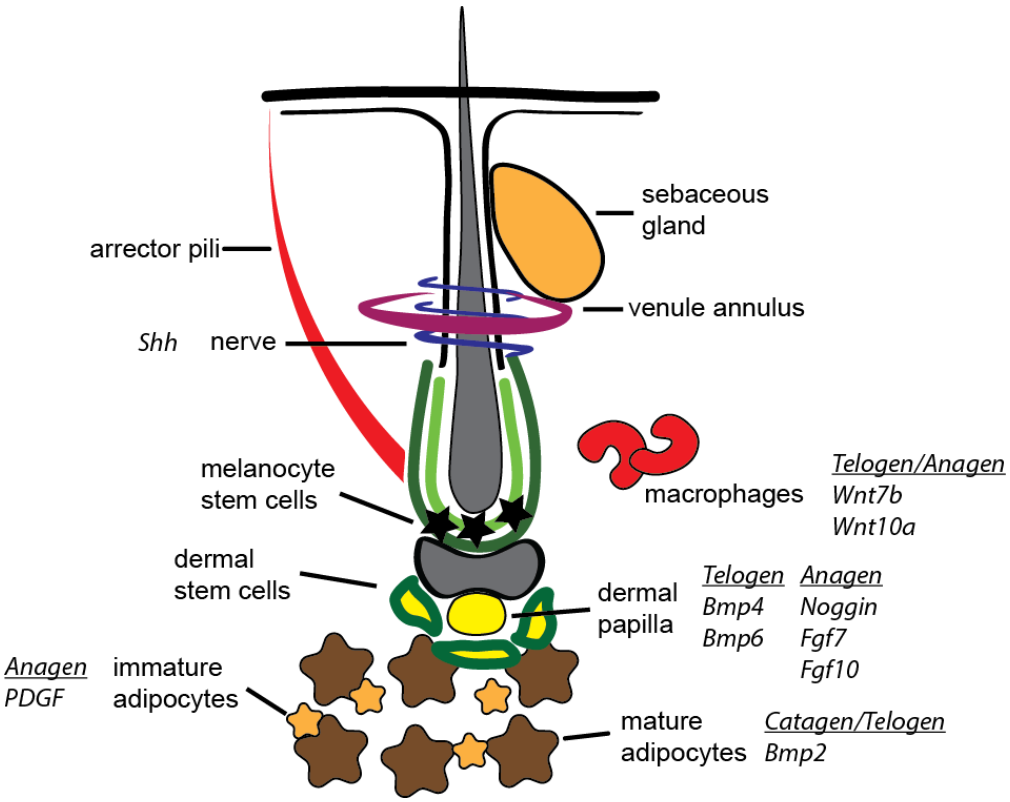



**Figure 2.2** Schematic of the non-epithelial environment surrounding the hair follicle. A number of these populations influence hair follicle homeostasis through both stimulatory and inhibitory signaling pathways.

*Dermal papilla*

As previously noted, the DP is a pocket of cells of dermal origin, which lies at the base of the HF, below the hair germ during telogen and encapsulated by the matrix during anagen. The DP has long been recognized as a critical signaling center regulating HF growth and quiescence. Loss of the DP by laser ablation arrests follicles at telogen, demonstrating the necessity of the DP for anagen progression [69]. Whereas, grafting of DP cells is sufficient for inducing hair growth in hairless skin [8]. Additionally, the number of DP cells effects the size of follicles, with smaller DPs producing shorter thinner hairs associated with aging [70].

FACS purification and gene expression profiling of the DP and its surrounding epithelial populations revealed potential crosstalk between the populations through Bmp, Wnt, Fgf, and Shh signaling, which is being borne out through subsequent studies [61]. The DP typically expresses high levels of HFSC quiescence promoting Bmp4 and Bmp6 [61]. However, to initiate hair germ proliferation at the start of anagen, the DP must also upregulate Bmp inhibitors such as *Noggin* and *Sostdc1* [71, 34]. Following anagen initiation, the DP continues to inhibit Bmp signaling to control the rate of hair growth [72]. The transcription factor *Sox2* in the DP has been demonstrated to maintain expression of Bmp inhibitor *Sostdc1*, and DP specific ablation of *Sox2* results in reduced hair growth due to inhibited migration of differentiating matrix progenitors into the hair shaft. Another Bmp inhibitor *Noggin* forms a regulatory loop with epithelial expressed Shh to both maintain DP function and hair growth [73]. Additionally, Notch-*Wnt5a* signaling from the DP promotes terminal differentiation of matrix progenitor cells [74].

In addition to Bmp signaling's role in hair cycling, Bmp is also essential within the DP for proper function [75]. When Bmp receptor 1a (Bmpr1a) is knocked out in the DP, the DP loses its ability to initiate hair growth when engrafted onto skin [75]. Similarly for Wnt signaling, HFs with β-catenin ablated in the DP are unable to enter anagen [76]. However, while these studies show that Bmp and Wnt are needed by the DP, the importance of these signals specifically from the epithelium remains unclear.

Although in general the signals seem to flow unidirectionally from the DP towards the bulge, the opposite direction is most likely also true. Early experiments showed that transplanting a bulge into hairless skin is sufficient to initiate formation of a new DP from neighboring dermal cells [11]. Moreover, when activated β-catenin is constitutively expressed in the epithelial bulge, it signals the surrounding dermal cells to form a new DP within each round of hair cycling [77]. These data suggest that in special conditions the bulge cells can regain some embryonic-like capabilities, where mesenchymal-epithelial crosstalk is thought to be essential for de novo HF formation [78, 7].

*Dermal fibroblasts and the dermal sheath*

Whereas much is known about the dermal papilla's relationship with the HF, relatively little is known about the HF's relationship with the surrounding dermal fibroblasts and the dermal sheath that outlines the hair. This is surprising because fibroblast have been long recognized as support cells for keratinocytes (skin epithelial cells) in cell culture [79]. These dermal cells are known to provide structural support for the epidermis and HF by producing collagen and other fibers for the extracellular matrix [80]. As

mentioned previously, embryonic HF morphogenesis involves the crosstalk between epithelial cells and dermal fibroblasts [78, 7]. In embryonic skin, aggregates of dermal fibroblasts, precursors to the DP, form and induce HF morphogenesis from the developing epidermis [7]. Wnt signaling is believed to be the first signal from the dermal aggregates responsible for hair induction. The transcription factor *Runx1* is highly but transiently expressed in the embryonic dermis and is essential for the maturation of emerging HFSCs and their long-term survival in the HF [39]. In this study lineage tracing using the *Runx1-CreER* knockin mouse showed that this population of *Runx1*-expressing cells are short-lived and do not survive past birth, perhaps explaining why new hairs are not induced in adulthood.

Nevertheless, dermal fibroblasts continue to influence HFSCs into adulthood. At the end of anagen and into telogen, fibroblasts express high levels of *Bmp4*, which helps to maintain quiescence in the bulge [21]. Signaling from the bulge to fibroblasts remains unexplored, although epithelial mutants have been shown to have an effect on the dermis. Epithelial activation of β-catenin not only induces dermal cells to form a new DP as stated previously [77], it also specifically reverts dermal fibroblasts around the isthmus to an embryonic state. These fibroblasts display increased proliferation and replaces mature collagen in the extracellular matrix with collagens subtypes found only in developing embryonic skin [81]. Why this effect is specifically seen in fibroblasts near the isthmus is unclear, but perhaps the isthmus maintains these cells in a more plastic state allowing them to be more easily reprogrammed.

A specialized layer of fibroblasts also forms the dermal sheath that lines the outside of the HF. The dermal sheath not only produces supportive collagen layers

around the HF, but the lower dermal sheath at the base of the HF serves as a niche for bipotent dermal SCs that can renew the dermal sheath and DP [82, 83]. The dermal sheath cycles through growth and destruction in parallel with the HF [83]. The dermal sheath surrounds the entire HF during anagen, but then undergoes apoptosis during catagen, leaving only the dermal SCs around the DP in catagen. Then during anagen, the dermal SCs proliferate to form a new dermal sheath as well as contributing some cells to the DP. How this synchrony between the dermal sheath and epithelial cells is achieved remains unknown, but it is suggestive of mesenchymal-epithelial crosstalk between the populations. Depletion of the dermal SCs causes delayed entry into anagen and reduced hair length, even though the average number of DP cells remained unchanged, presumably due to compensating proliferation within the DP [83]. This suggests that either the dermal SC derived DP cells serve an additional stimulatory function independent of the resident DP cells, or perhaps the dermal sheath promotes proliferation. Additionally, the bulge has been shown to upregulate connective tissue growth factor, CTGF [13], which may stimulate recruitment of dermal sheath cells to around the HF. Overall, the relationship between dermal fibroblasts, particularly the dermal sheath, and HFSCs is poorly understood, and additional experiments are necessary to determine if these fibroblasts have additional functions in supporting the HFSC niche beyond formation of the extracellular matrix. In addition, it will be interesting to see if the extracellular matrix around the HF is heterogeneous in nature, perhaps dictating some of the spatial heterogeneity observed within the stem cell niche.

*Adipocytes*

Below the HF lies an intradermal adipocyte layer: the skin's own fat cells. Immature adipocyte progenitors, derived from the dermal resident adipocyte SCs are necessary and sufficient to drive the proliferation of HFSCs [84]. Using mouse mutants or pharmacological treatments to target various stages of adipogenesis, Festa *et al*., were able to establish the necessity of the adipocyte progenitor cells for bulge HFSC activation and anagen progression. The adipocyte progenitor cells seem to exert their influence on HFSCs through platelet derived growth factor (PDGF) signaling, which is received by the DP, although a specific knockout of PDGF in adipocytes has yet to be performed. During anagen, this adipocyte layer doubles in thickness from adipogenesis when the progenitor cells generate mature terminally differentiated adipocytes, paralleling the proliferation and differentiation of HFSCs [84]. The mature adipocytes generated in the intradermal adipocyte layer show periodic expression of the inhibitory signal *Bmp2* [21]. *Bmp2* expression is high in late catagen and early telogen but is absent in late telogen. BMP2 from adipocytes and BMP4 from the DP and dermal fibroblasts might be responsible for establishing a refractory period at the beginning of telogen. During this refractory period, the threshold for bulge SC activation is high, and even injury by hair plucking cannot induce anagen. Quiescence is maintained until Bmp levels are again lowered in the surrounding adipocytes and dermis, allowing anagen to be initiated [21].

Signaling from epithelial cells to adypocites remains unknown, but epithelial mutations that block hair cycle progression also block adipogenesis [30, 29, 27]. This strongly suggests that adipocytes wait for a response signal from the epithelial SCs

before initiating adipogenesis. It is unclear if the first step in activation is when adipocyte SCs generate the progenitors that in turn must signal to epithelial cells, or whether the epithelial signals are required prior to this step in adipogenesis. In other words, it is currently unclear what the primary signal is that sets in motion the crosstalk between fat and epithelial cells in the skin. In addition, it is tempting to speculate that as the HFSC begin to proliferate they signal to fat cell for the imminent necessity of vast sources of energy, which are then stored as lipids in mature adipocytes in preparation for rapid hair production/elongation. These lipids would then be subsequently delivered to the matrix cells, thus fueling the generation of the new hair shaft.

*Blood Vessels*

Nutrients are delivered to HF and epidermis through a microvascular network that surrounds most of the follicle and undergoes extensive remodeling and angiogenesis throughout the hair cycle [85]. Angiogenesis significantly increases during early anagen, perhaps to support the increased oxygen demands of the rapidly proliferating matrix. Consistent with this possibility, pharmaceutically inhibiting angiogenesis with the drug TNP-470 is capable of delaying anagen onset [85], whereas transgenic overexpression in epithelial cells of vascular endothelial growth factor (VEGF) increases angiogenesis and causes accelerated hair growth [86]. Transplantation assays demonstrate that the bulge and anagen matrix (but not the DP) are capable of stimulating angiogenesis [87, 88], however the signaling mechanism remains unknown. This suggests that rather than associating randomly with the HF, the

microvascular network may be directed from the epithelial cells to form around these compartments.

Indeed, three-dimensional confocal imaging has revealed that a ring of venules permanently wraps around the HF isthmus, a compartment between the bulge and sebaceous gland that contains a distinct, rapidly cycling, SC population that renews the upper portion of the HF [89]. HFs can be formed *de novo* in mouse skin reconstituted from primary keratinocytes and dermal fibroblasts, and when transplanted the association between the isthmus and venules is reestablished. Consistent with recruitment to *de novo* HFs, the isthmus expresses Egfl6, a member of the epidermal growth factor (EGF) superfamily known to promote endothelial migration and angiogenesis [89]. As yet, whether or not the vascular ring has a role in HFSC function remains unknown. It is interesting to consider the possibility that blood vessels can contribute to oxygen availability and consumption, which in turn would support oxidative phosphorylation, and the generation of reactive oxygen species (ROS). ROS is a hallmark of cells undergoing rapid proliferation and has been shown to act as a differentiation signal in other SC systems [90]. In the HF, ROS signaling is necessary during anagen for matrix cell proliferation and survival and loss of mitochondrial ROS causes follicles to prematurely enter catagen [91].

It has been hypothesized that tissue stem cells in general maintain their quiescence and are protected against oxidative stress by residing in a low oxygen environment [92]. As with the neural, hematopoietic, and mesenchymal niches [92], expression of hypoxia markers suggests that the bulge may also be a low oxygen environment [93]. The blood vessels associated with the upper bulge are venous in

nature, meaning the blood is low in oxygen and nutrients, and may therefore establish the hypoxic microenvironment [89]. This suggests that the presence of blood vessels in niche is not only important to provide nutrients to stem cells but also to create gradients of oxygen tension that could play an important role in regulating cell fate. It was demonstrated in the neural stem cell (NSC) niche that hypoxic stimuli lead to expression of hypoxia inducible factor 1 (HIF-1) [94]. HIF-1 in NSCs promotes stemness by upregulating expression of Notch target genes and cyclin D1 to promote cell cycle progression and self-renewal [95]. In the future, it will be interesting to examine the relationship between the blood vessel microenvironment, hypoxia, and HFSC fate. It will be particularly interesting to determine if signals from the bulge are capable of recruiting additional blood vessels in response to the demands of the hair cycle, and whether blood vessels are indeed essential for HF oxygen homeostasis or have additional unknown functions for HFSC survival and cell fate.

*Neurons*

Mechanosensory nerves wrap around the HF isthmus and make contacts with the upper bulge allowing the hair to sense when it is touched [96]. Additionally, these nerves form a perineural microenvironment that displays increased Shh signaling [97]. The perineural microenvironment establishes a distinct domain at the bottom of the isthmus directly above the bulge that is K15 negative and expresses the HH mediating transcription factor *Gli1*. Although hypothesized to contribute to bulge HFSC activity, removing the Shh microenvironment by denervation had no affect on the bulge or normal HF homeostasis. Denervation did however inhibit the ability of isthmus SCs to

contribute long-term to epidermal wounds, suggesting a possible role for Shh signaling in this process [97]. While removing the nerves does not affect hair cycling, the converse experiment where nerves are augmented has not been performed yet. It also remains unclear how nerve terminations are guided to the HF, but the bulge cells produce high levels of brain derived growth factor (BDNF) as well as guiding and repelling signals such as ephrins [13]. These signals might be important in patterning the nerve terminations around the HF.

*Immune cells*

Hair follicles are one of the few sites in the body, along with the brain and the eye, of relative immune privilege. In particular, the anagen bulge and matrix have decreased major histocompatibility complex (MHC) class I expression and increased expression of local immunosuppressants [98, 99]. Although the purpose of establishing immune privilege during anagen remains unclear, its maintenance is critical as loss of immune privilege and autoimmune attacks on the matrix and the bulge are associated with alopecia [100].

Interestingly, macrophages are present in the perifollicular environment and have been found to contribute to the activation of HFSCs during wounding and homeostasis [101, 102]. Following wounding, apoptosis signal-regulating kinase 1 (ASK1), a MAP3K family member, is upregulated at the wound site to recruit and activate macrophages [101]. Failure to recruit macrophages to the wound in *ASK1* mutant mice causes hair growth to be delayed at the wound. Furthermore, transplantation of activated macrophages is sufficient to induce hair growth.

Macrophages may also contribute to HFSC activation in normal homeostasis. During telogen, macrophages residing around the follicle upregulate *Wnt7b* and *Wnt10a* into the perifollicular environment, however the Wnt ligands are not released from the macrophages unless they undergo apoptosis [102]. The signal that triggers macrophage apoptosis is unknown, but at the end of telogen, much of the resident macrophage population undergoes apoptosis, releasing the Wnt ligands that in turn promote HF entry into anagen. Ablation of the macrophages pharmacologically or with genetically inducible diphtheria toxin mice is also sufficient to release the Wnt ligands and initiate entry into anagen [102]. This is seemingly at odds with the previous finding that transplantation of macrophages is also sufficient for anagen induction, although it is possible the transplanted macrophages must also undergo apoptosis to affect hair growth.

Mice lacking γδ T cells have also been shown to have shortened telogen stages [103], suggesting immune cells in general may have more influence on the hair than just protection from pathogens. Perhaps loss of immune cells to establish immune privilege is necessary for anagen entry, and the apoptotic release of Wnt ligands seen in macrophages is a signal to the HF that this has been achieved. Conversely, it is unclear whether return of immune cells after anagen is necessary. During anagen, the HF actively inhibits immune cells by the local production of immunosuppressants such as macrophage migration inhibitory factor [100], however it would be interesting to see if the bulge expresses factors to attract macrophages and other immune cells back following anagen.

*Arrector pili muscle*

The arrector pili muscle (APM) attaches to the HF at the bulge throughout the hair cycle and is responsible for piloerection, which aids in thermoregulation. The attachment of the APM at the bulge depends on the extracellular matrix protein nephronectin, which is secreted by bulge cells into the underlying basement membrane [104]. In cell culture, dermal cells expressing the nephronectin receptor α8-integrin, which were used as surrogate for muscle cells, attached to extracellular nephronectin and in turn stimulated the expression of smooth muscle markers. These data suggested that bulge epithelial cells utilize nephronectin to promote muscle differentiation and perhaps dictate where the APM attaches to the follicle. Full nephronectin knockout caused the APM attachment site to shift upwards to the isthmus, with α8-integrin instead interacting with another nephronectin family member EGFL6. Full knockout of α8-integrin causes the APM to randomly attach either to the bulge or the EGFL6+ isthmus. Despite the loss of APM contact at the bulge, there appeared to be no change in the bulge or HF morphology [104]. However, it is unknown if augmenting differentiation of APM at the bulge has any effect.

In summary, the bulge SCs seem to be involved with their neighboring cells in distinct manners, following different rules of interaction. First, the bulge SCs stay intimately connected with their direct progeny in the inner layer and depend on them for their survival, quiescence, and to inhibit differentiation. Second, the bulge SCs seem to receive instructions from the DP regarding their activation and differentiation, but we have yet to learn about any instructive responses from epithelial cells towards the DP. With that said, the bulge is capable, in the right setting to build its own DP

from neighboring dermal cells. Similarly, this is seen with the APM, blood vessels, and nerves, demonstrating the bulge is equipped with the mechanisms necessary to recruit its neighbors for proper HF physiology. In the case of the APM and nerves, this recruitment occurs without receiving feedback signals and without any clear effect on HFSC status or hair growth, while for dermal cells, blood vessels, and fat cells a meaningful cross-talk is apparent.

### *2.9 Maintenance and aging*

Throughout their lifetime, SCs are prone to acquiring mutations that could lead to disease and cancer. Niches are commonly viewed as special microenvironments, protecting SCs from assaults such as genomic damage or proliferating and differentiating signals, so that they can survive long-term and maintain their potential to support the organism throughout its lifetime (**Figure 2.3**). A low oxygen environment helps the SCs avoid oxidative stress and promotes quiescence. Also, high oxygen and ROS are known signals for differentiation [91, 90], which SCs must avoid if they are to maintain their potential long-term. As suggested above (see above section on blood vessels) the bulge SCs might not only be equipped with the right environment for maintenance but may in fact actively contribute to creating that environment around their niche.

Bulge quiescence is not only an essential mechanism for ensuring sufficient SCs persist for long-term hair growth. Not surprisingly, quiescence also proves to be a valuable method of tumor suppression. While bulge cells may serve as cancer initiating cells (discussed further in the next section), it has recently been shown that

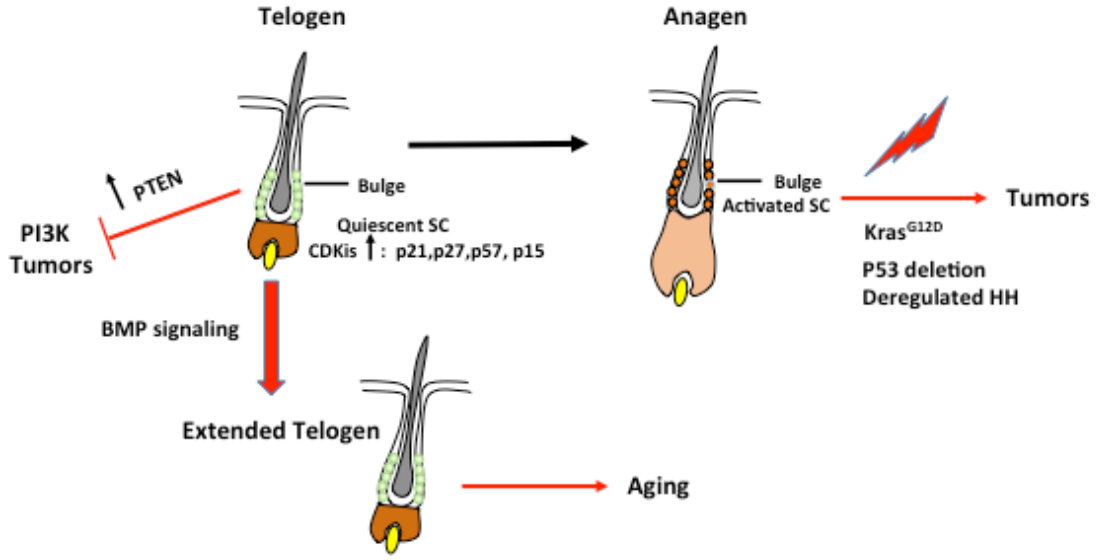



**Figure 2.3** Quiescence maintains the stem cell pool by preventing their premature exhaustion. Expression of cyclin-dependent kinase inhibitors (CDKIs) such as *p21*, *p27*, *p57*, and *p15* promote quiescence. The extended telogen appears to be a result of increased sensitivity of HFSCs to Bmp signaling which leads to aging. Quiescence also acts as a method of tumor suppression, quiescent bulge cells are resistant to *KrasG12D* dependent tumors *via* tumor suppressor Pten, which inhibit the PI (3) K pathway. Bulge tumorigenesis only occurred when *KrasG12D* was induced during the telogen-to-anagen transition i.e. upon stem cell activation. Activated HFSCs are the origin of different cancers caused by genetic alterations such as deregulated HH signaling, conditional activation of oncogenic *Kras* and deletion of *p53* in K15+ bulge cells but not from matrix progenitor cells.

normal activation of the HFSCs is in fact required for tumorigenesis [105]. Using an inducible oncogenic *Kras* mutation ($Kras^{G12D}$), White *et al*. demonstrated that bulge hyperplasia and tumorigenesis only occurred when $Kras^{G12D}$ was induced during the telogen-to-anagen transition, the period when bulge cells are activated to divide for self-renewal. During both telogen and late anagen/catagen, the bulge cells are quiescent and unable to initiate tumorigenesis. This quiescence-associated resistance to $Kras^{G12D}$ is not simply a matter of not proliferating. Rather, quiescent SCs depend on *Pten* to inhibit the phosphatidylinositol-3-OH kinase (PI(3)K) pathway downstream of *Ras*.

Since the bulge is located in the proximity of the skin surface, HFSCs are susceptible to damage from UV radiation. Therefore, the HFSCs must have been endowed with their own intrinsic properties allowing them to maintain their integrity. In contrast with embryonic SCs, which readily undergo apoptosis in response to DNA damage, it has been shown that HFSCs resist apoptosis in favor of repairing the damage [106]. Bulge cells express higher levels of the anti-apoptotic gene *Bcl-2* compared to the rest of the epidermis, which helps HFSCs resist apoptosis following exposure to ionizing radiation [107]. They also express high levels of the key non-homologous end joining repair pathway protein DNA-PK, allowing HFSCs to repair the damage more rapidly than the rest of the epidermal populations.

In addition to specialized repair, the bulge also expresses a number of transcription factors necessary to maintain stemness [27, 108, 109]. Two transcription factors, *Sox9* and *Lhx2* have been found to maintain the stemness of bulge cells by promoting quiescence and inhibiting differentiation [109, 108, 110]. *Sox9* is expressed

early on in embryonic HF morphogenesis and is necessary for SC specification [111, 112]. When *Sox9* is ablated from the adult HF, the bulge cells are unable to return to quiescence after activation in anagen and begin terminally differentiating into epidermal cells [109]. Similarly, epithelial *Lhx2* knockout mice display enhanced bulge proliferation as well as abnormal differentiation into sebaceous gland cells [108, 110]. On the other hand, transcription factor *Tbx1* acts to allow HFSCs to exit quiescence and self renew by suppressing Bmp signaling during anagen [27]. As such, epithelial *Tbx1* knockout follicles have prolonged quiescence and challenging the SCs with depilation induced activation results in depletion of the bulge and dormant HFs resembling those found during aging.

Aging is associated with a decline in maintenance for various tissues. In hair, aging is associated with greying, extended quiescence, hair shaft thinning, and decreased density. Age related hair graying is caused by depletion of melanocyte SCs due to apoptosis and premature differentiation [113, 49]. One of the hallmarks of aging that leads to a decline in SC functionality and senescence is shortened telomeres [114]. Since telomere shortening is a consequence of DNA replication, maintaining quiescence is likely important for delaying telomere shortening. Additionally, bulge cells express telomerase, a specialized DNA polymerase that extends telomeres [115, 116], and therefore maintain longer telomeres than the rest of the HF [117]. However, telomerase is insufficiently expressed in adult SCs to maintain telomere length, and indeed bulge cells display significantly shorter telomeres in aged mice than in young mice [117]. Bulge cells that reach critically short telomere length lose their ability to activate and leave the niche to contribute to hair growth [115].

The hair cycle also displays age-related changes, characterized by extended periods of telogen [118]. These extended telogens appear to be a result of increased sensitivity of HFSCs to Bmp signaling. Not only do aged HFs resist homeostatic cycling, but also, even forced entry into anagen by depilation is significantly delayed in older mice. Further, following depilation, transcription profiles of aged anagen HFSCs more closely resemble HFSCs from young telogen than young anagen. This age-related change in behavior and shortening of telomeres may reflect a shift in priorities for HFSCs, as hair growth and regeneration is compromised in favor of the tumor suppressing benefits of an extended quiescence.

### *2.10 Bulge as the origin of skin cancers*

While the long-term maintenance of SCs is necessary for tissue regeneration, their extended lifespan also puts them at an increased risk of acquiring oncogenic mutations to initiate tumors and cancers [119]. Genetic lineage tracing has placed the bulge as the cell of origin for the two most common forms of cancer: skin basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCCs are characterized by deregulation of Hedgehog (HH) signaling. Using a conditional Smoothened mutant (*SmoM2*) to constitutively activate HH signaling and induce BCC formation, genetic lineage tracing showed that BCCs originate from the infundibulum and interfollicular epidermis [120]. Conditionally activating HH in either the bulge or matrix populations with lineage specific *Cre* reporter expression was unable to initiate BCCs. However, in a different BCC model utilizing a deletion of the HH inhibitor *Ptch1* and exposure to ionizing radiation gave rise to BCCs predominantly originating from K15+ bulge

SCs [121]. These opposing models likely represent two different subtypes of BCCs. A further complication in the identification of the cell of origin for skin cancer may lie in the recruitment of bulge SCs to the interfollicular epidermis in response to injury [13, 43]. Indeed, in HH activation by *Ptch1* deletion or *Gli1* overexpression, wounding promotes the contribution of bulge cells to tumor formation [122].

Bulge SCs have also been shown to act as the cell of origin for SCCs [123, 124]. Lineage tracing has shown that SCCs could be initiated from the conditional activation of oncogenic Kras and deletion of *p53* in K15+ bulge cells but not from matrix progenitor cells [123]. A bulge origin for SCCs has also been demonstrated by lineage tracing in tumors induced chemically with DMBA/TPA [125]. In addition to originating from HFSCs, SCCs have also been demonstrated to utilize the intrinsic SC machinery to promote tumorigenicity, such as with the HFSC transcription factor *Runx1* [31, 124]. Intriguingly, while *Runx1* is dispensable for HFSC survival and long-term function [30], it is strictly required for both tumor initiation and maintenance in DMBA/TPA and *Kras* induced mice [124]. Identification of the bulge as cancer initiating cells provides an important starting point for determining the genetic lesions involved in tumor initiation. Importantly, beyond extending our understanding of cancer development, the gene signature of the bulge may help to identify key pathways or targets, such as *Runx1*, for new therapeutic approaches.

### *2.11 Conclusion*

As our knowledge of the HF progresses, our conception of the bulge has expanded far beyond simply a storehouse of SCs awaiting activation. Regulation of HFSC behavior

is an intricate process that involves the bulge acting as a central signaling hub, integrating signals from the rest of the HF and its non-epithelial neighbors. Normal hair growth not only depends on the DP, but also on signaling from adipocytes and macrophages. Additionally, the bulge does not simply receive signals, but also transmits signals to attract blood vessels, neurons, and muscle cells to form a complete follicular unit. As our tools to manipulate and examine the bulge and its neighbors expand, it will be exciting to discover what new interactions can be unveiled.

Examination of SC self-renewal and differentiation has been and will be an area of intense interest, bringing us ever closer to the goal of tissue regeneration. However, of equal importance is SC quiescence. A main function of the bulge niche environment is concerned with enforcing HFSC quiescence, which is as an important mechanism for genomic maintenance and tumor suppression. Quiescent HFSCs are not just waiting idly in their niche. Quiescence is likely not just a “resting" phase but rather a more dynamic process than previously believed, with HFSCs preparing for the next stage of activation. Further investigation of quiescence is needed to better understand its role in SC maintenance and disease states.

# REFERENCES


1. Müller-Röver S, Handjiski B, van der Veen C, Eichmüller S, Foitzik K, McKay IA et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. The Journal of investigative dermatology. 2001;117(1):3-15. doi:10.1046/j.0022-202x.2001.01377.x.
2. Waghmare SK, Bansal R, Lee J, Zhang YV, McDermitt DJ, Tumbar T. Quantitative proliferation dynamics and random chromosome segregation of hair follicle stem cells. The EMBO journal. 2008;27(9):1309-20. doi:10.1038/emboj.2008.72.
3. Zhang YV, Cheong J, Ciapurin N, McDermitt DJ, Tumbar T. Distinct self-renewal and differentiation phases in the niche of infrequently dividing hair follicle stem cells. Cell Stem Cell. 2009;5(3):267-78. doi:10.1016/j.stem.2009.06.004.
4. Cotsarelis G. Epithelial stem cells: a folliculocentric view. J Invest Dermatol. 2006;126(7):1459-68. doi:10.1038/sj.jid.5700376.
5. Fuchs E. Scratching the surface of skin development. Nature. 2007;445(7130):834-42. doi:10.1038/nature05659.
6. Rompolas P, Greco V. Stem cell dynamics in the hair follicle niche. Semin Cell Dev Biol. 2014;25-26:34-42. doi:10.1016/j.semcdb.2013.12.005.
7. Yang C-C, Cotsarelis G. Review of hair follicle dermal cells. Journal of dermatological science. 2010;57(1):2-11. doi:10.1016/j.jdermsci.2009.11.005.
8. Sennett R, Rendl M. Mesenchymal-epithelial interactions during hair follicle morphogenesis and cycling. Semin Cell Dev Biol. 2012;23(8):917-27. doi:10.1016/j.semcdb.2012.08.011.

9. Goldstein J, Horsley V. Home sweet home: skin stem cell niches. Cell Mol Life Sci. 2012;69(15):2573-82. doi:10.1007/s00018-012-0943-3.

10. Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell. 1990;61(7):1329-37.

11. Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell. 2001;104(2):233-45.

12. Claudinot S, Nicolas M, Oshima H, Rochat A, Barrandon Y. Long-term renewal of hair follicles from clonogenic multipotent stem cells. Proc Natl Acad Sci U S A. 2005;102(41):14677-82. doi:10.1073/pnas.0507250102.

13. Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M et al. Defining the epithelial stem cell niche in skin. Science. 2004;303(5656):359-63. doi:10.1126/science.1092436.

14. Trempus CS, Morris RJ, Bortner CD, Cotsarelis G, Faircloth RS, Reece JM et al. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J Invest Dermatol. 2003;120(4):501-11. doi:10.1046/j.1523-1747.2003.12088.x.

15. Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G. The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. Journal of Cell Science. 1998;111 ( Pt 21):3179-88.

16. Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S et al. Capturing and profiling adult hair follicle stem cells. Nat Biotechnol. 2004;22(4):411-7. doi:10.1038/nbt950.

17. Lee J, Tumbar T. Hairy tale of signaling in hair follicle development and cycling. Seminars in cell & developmental biology. 2012;23(8):906-16. doi:10.1016/j.semcdb.2012.08.003.

18. Hsu YC, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. Cell. 2011;144(1):92-105. doi:10.1016/j.cell.2010.11.049.

19. Fuchs E. The tortoise and the hair: slow-cycling cells in the stem cell race. Cell. 2009;137(5):811-9. doi:10.1016/j.cell.2009.05.002.

20. Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. Science (New York, NY). 2010;327(5965):542-5. doi:10.1126/science.1180794.

21. Plikus MV, Mayer JA, de la Cruz D, Baker RE, Maini PK, Maxson R et al. Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature. 2008;451(7176):340-4. doi:10.1038/nature06457.

22. Kobielak K, Stokes N, de la Cruz J, Polak L, Fuchs E. Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. Proc Natl Acad Sci U S A. 2007;104(24):10063-8. doi:10.1073/pnas.0703004104.

23. Horsley V, Aliprantis AO, Polak L, Glimcher LH, Fuchs E. NFATc1 balances quiescence and proliferation of skin stem cells. Cell. 2008;132(2):299-310. doi:10.1016/j.cell.2007.11.047.

24. Takeda N, Jain R, Leboeuf MR, Padmanabhan A, Wang Q, Li L et al. Hopx expression defines a subset of multipotent hair follicle stem cells and a progenitor population primed to give rise to K6+ niche cells. Development. 2013;140(8):1655-64. doi:10.1242/dev.093005.

25. Castilho RM, Squarize CH, Chodosh LA, Williams BO, Gutkind JS. mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell. 2009;5(3):279-89. doi:10.1016/j.stem.2009.06.017.

26. Nakajima T, Inui S, Fushimi T, Noguchi F, Kitagawa Y, Reddy JK et al. Roles of MED1 in quiescence of hair follicle stem cells and maintenance of normal hair cycling. J Invest Dermatol. 2013;133(2):354-60. doi:10.1038/jid.2012.293.

27. Chen T, Heller E, Beronja S, Oshimori N, Stokes N, Fuchs E. An RNA interference screen uncovers a new molecule in stem cell self-renewal and long-term regeneration. Nature. 2012;485(7396):104-8. doi:10.1038/nature10940.

28. Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell. 2004;118(5):635-48. doi:10.1016/j.cell.2004.08.012.

29. Lee J, Hoi CS, Lilja KC, White BS, Lee SE, Shalloway D et al. Runx1 and p21 synergistically limit the extent of hair follicle stem cell quiescence in vivo. Proc Natl Acad Sci U S A. 2013;110(12):4634-9. doi:10.1073/pnas.1213015110.

30. Osorio KM, Lee SE, McDermitt DJ, Waghmare SK, Zhang YV, Woo HN et al. Runx1 modulates developmental, but not injury-driven, hair follicle stem cell activation. Development (Cambridge, England). 2008;135(6):1059-68. doi:10.1242/dev.012799.

31. Hoi CS, Lee SE, Lu SY, McDermitt DJ, Osorio KM, Piskun CM et al. Runx1 directly promotes proliferation of hair follicle stem cells and epithelial tumor formation in mouse skin. Mol Cell Biol. 2010;30(10):2518-36. doi:10.1128/MCB.01308-09.

32. Devgan V, Mammucari C, Millar SE, Brisken C, Dotto GP. p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. Genes Dev. 2005;19(12):1485-95. doi:10.1101/gad.341405.

33. Pippa R, Espinosa L, Gundem G, Garcia-Escudero R, Dominguez A, Orlando S et al. p27Kip1 represses transcription by direct interaction with p130/E2F4 at the promoters of target genes. Oncogene. 2012;31(38):4207-20. doi:10.1038/onc.2011.582.

34. Greco V, Chen T, Rendl M, Schober M, Pasolli HA, Stokes N et al. A two-step mechanism for stem cell activation during hair regeneration. Cell Stem Cell. 2009;4(2):155-69. doi:10.1016/j.stem.2008.12.009.

35. Ito M, Kizawa K, Hamada K, Cotsarelis G. Hair follicle stem cells in the lower bulge form the secondary germ, a biochemically distinct but functionally equivalent progenitor cell population, at the termination of catagen. Differentiation. 2004;72(9-10):548-57. doi:10.1111/j.1432-0436.2004.07209008.x.

36. Lee SE, Sada A, Zhang M, McDermitt DJ, Lu SY, Kemphues KJ et al. High Runx1 levels promote a reversible, more-differentiated cell state in hair-follicle stem cells during quiescence. Cell Rep. 2014;6(3):499-513. doi:10.1016/j.celrep.2013.12.039.

37. Zhang YV, White BS, Shalloway DI, Tumbar T. Stem cell dynamics in mouse hair follicles: a story from cell division counting and single cell lineage tracing. Cell Cycle. 2010;9(8):1504-10.

38. Rompolas P, Mesa KR, Greco V. Spatial organization within a niche as a determinant of stem-cell fate. Nature. 2013;502(7472):513-8. doi:10.1038/nature12602.

39. Osorio KM, Lilja KC, Tumbar T. Runx1 modulates adult hair follicle stem cell emergence and maintenance from distinct embryonic skin compartments. J Cell Biol. 2011;193(1):235-50. doi:10.1083/jcb.201006068.

40. Hsu YC, Li L, Fuchs E. Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. Cell. 2014;157(4):935-49. doi:10.1016/j.cell.2014.02.057.

41. Barry ER, Camargo FD. The Hippo superhighway: signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. Curr Opin Cell Biol. 2013;25(2):247-53. doi:10.1016/j.ceb.2012.12.006.

42. Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell. 2000;102(4):451-61.

43. Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ et al. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nat Med. 2005;11(12):1351-4. doi:10.1038/nm1328.

44. Horsley V, O'Carroll D, Tooze R, Ohinata Y, Saitou M, Obukhanych T et al. Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland. Cell. 2006;126(3):597-609. doi:10.1016/j.cell.2006.06.048.

45. Snippert HJ, Haegebarth A, Kasper M, Jaks V, van Es JH, Barker N et al. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science (New York, NY). 2010;327(5971):1385-9. doi:10.1126/science.1184733.

46. Jensen KB, Collins CA, Nascimento E, Tan DW, Frye M, Itami S et al. Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. Cell Stem Cell. 2009;4(5):427-39. doi:10.1016/j.stem.2009.04.014.

47. Jaks V, Barker N, Kasper M, van Es JH, Snippert HJ, Clevers H et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet. 2008;40(11):1291-9. doi:10.1038/ng.239.

48. Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M et al. Dominant role of the niche in melanocyte stem-cell fate determination. Nature. 2002;416(6883):854-60. doi:10.1038/416854a.

49. Nishimura EK. Melanocyte stem cells: a melanocyte reservoir in hair follicles for hair and skin pigmentation. Pigment Cell & Melanoma Research. 2011;24(3):401-10. doi:10.1111/j.1755-148X.2011.00855.x.

50. Tanimura S, Tadokoro Y, Inomata K, Binh NT, Nishie W, Yamazaki S et al. Hair follicle stem cells provide a functional niche for melanocyte stem cells. Cell Stem Cell. 2011;8(2):177-87. doi:10.1016/j.stem.2010.11.029.

51. Chang CY, Pasolli HA, Giannopoulou EG, Guasch G, Gronostajski RM, Elemento O et al. NFIB is a governor of epithelial-melanocyte stem cell behaviour in a shared niche. Nature. 2013;495(7439):98-102. doi:10.1038/nature11847.

52. Rabbani P, Takeo M, Chou W, Myung P, Bosenberg M, Chin L et al. Coordinated Activation of Wnt in Epithelial and Melanocyte Stem Cells Initiates Pigmented Hair Regeneration. Cell. 2011;145(6):941-55. doi:10.1016/j.cell.2011.05.004.

53. Reddy S, Andl T, Bagasra A, Lu MM, Epstein DJ, Morrisey EE et al. Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. Mech Dev. 2001;107(1-2):69-82.

54. Hsu Y-C, Li L, Fuchs E. Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. Cell. 2014;157(4):935-49. doi:10.1016/j.cell.2014.02.057.

55. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development. 1999;126(20):4557-68.

56. Merrill BJ, Gat U, DasGupta R, Fuchs E. Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. Genes Dev. 2001;15(13):1688-705. doi:10.1101/gad.891401.

57. Kaufman CK, Zhou P, Pasolli HA, Rendl M, Bolotin D, Lim K-C et al. GATA-3: an unexpected regulator of cell lineage determination in skin. Genes & Development. 2003;17(17):2108-22. doi:10.1101/gad.1115203.

58. Legué E, Nicolas J-F. Hair follicle renewal: organization of stem cells in the matrix and the role of stereotyped lineages and behaviors. Development (Cambridge, England). 2005;132(18):4143-54. doi:10.1242/dev.01975.

59. Legué E, Sequeira I, Nicolas J-F. Hair follicle renewal: authentic morphogenesis that depends on a complex progression of stem cell lineages. Development (Cambridge, England). 2010;137(4):569-77. doi:10.1242/dev.044123.

60. Sequeira I, Nicolas J-F. Redefining the structure of the hair follicle by 3D clonal analysis. Development (Cambridge, England). 2012;139(20):3741-51. doi:10.1242/dev.081091.

61. Rendl M, Lewis L, Fuchs E. Molecular dissection of mesenchymal-epithelial interactions in the hair follicle. PLoS Biol. 2005;3(11):e331. doi:10.1371/journal.pbio.0030331.

62. Ellis T, Gambardella L, Horcher M, Tschanz S, Capol J, Bertram P et al. The transcriptional repressor CDP (Cutl1) is essential for epithelial cell differentiation of the lung and the hair follicle. Genes & Development. 2001;15(17):2307-19. doi:10.1101/gad.200101.

63. Cai J, Lee J, Kopan R, Ma L. Genetic interplays between Msx2 and Foxn1 are required for Notch1 expression and hair shaft differentiation. Dev Biol. 2009;326(2):420-30. doi:10.1016/j.ydbio.2008.11.021.

64. Hwang J, Mehrani T, Millar SE, Morasso MI. Dlx3 is a crucial regulator of hair follicle differentiation and cycling. Development (Cambridge, England). 2008;135(18):3149-59. doi:10.1242/dev.022202.

65. Weiner L, Han R, Scicchitano BM, Li J, Hasegawa K, Grossi M et al. Dedicated epithelial recipient cells determine pigmentation patterns. Cell. 2007;130(5):932-42. doi:10.1016/j.cell.2007.07.024.

66. Ma L, Liu J, Wu T, Plikus M, Jiang TX, Bi Q et al. 'Cyclic alopecia' in Msx2 mutants: defects in hair cycling and hair shaft differentiation. Development. 2003;130(2):379-89.

67. Jave-Suarez LF, Winter H, Langbein L, Rogers MA, Schweizer J. HOXC13 is involved in the regulation of human hair keratin gene expression. J Biol Chem. 2002;277(5):3718-26. doi:10.1074/jbc.M101616200.

68. Wang AB, Zhang YV, Tumbar T. Gata6 promotes hair follicle progenitor cell renewal by genome maintenance during proliferation. EMBO J. 2016. doi:10.15252/embj.201694572.

69. Rompolas P, Deschene ER, Zito G, Gonzalez DG, Saotome I, Haberman AM et al. Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. Nature. 2012;487(7408):496-9. doi:10.1038/nature11218.

70. Chi W, Wu E, Morgan BA. Dermal papilla cell number specifies hair size, shape and cycling and its reduction causes follicular decline. Development (Cambridge, England). 2013;140(8):1676-83. doi:10.1242/dev.090662.

71. Botchkarev VA, Botchkareva NV, Nakamura M, Huber O, Funa K, Lauster R et al. Noggin is required for induction of the hair follicle growth phase in postnatal skin. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2001;15(12):2205-14. doi:10.1096/fj.01-0207com.

72. Clavel C, Grisanti L, Zemla R, Rezza A, Barros R, Sennett R et al. Sox2 in the dermal papilla niche controls hair growth by fine-tuning BMP signaling in

differentiating hair shaft progenitors. Dev Cell. 2012;23(5):981-94. doi:10.1016/j.devcel.2012.10.013.

73. Woo W-M, Zhen HH, Oro AE. Shh maintains dermal papilla identity and hair morphogenesis via a Noggin-Shh regulatory loop. Genes & Development. 2012;26(11):1235-46. doi:10.1101/gad.187401.112.

74. Hu B, Lefort K, Qiu W, Nguyen B-C, Rajaram RD, Castillo E et al. Control of hair follicle cell fate by underlying mesenchyme through a CSL-Wnt5a-FoxN1 regulatory axis. Genes & Development. 2010;24(14):1519-32. doi:10.1101/gad.1886910.

75. Rendl M, Polak L, Fuchs E. BMP signaling in dermal papilla cells is required for their hair follicle-inductive properties. Genes Dev. 2008;22(4):543-57. doi:10.1101/gad.1614408.

76. Enshell-Seijffers D, Lindon C, Kashiwagi M, Morgan BA. beta-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. Dev Cell. 2010;18(4):633-42. doi:10.1016/j.devcel.2010.01.016.

77. Gat U, DasGupta R, Degenstein L, Fuchs E. De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell. 1998;95(5):605-14.

78. Millar SE. Molecular Mechanisms Regulating Hair Follicle Development. Journal of Investigative Dermatology. 2002;118(2):216-25. doi:doi:10.1046/j.0022-202x.2001.01670.x.

79. Barrandon Y, Li V, Green H. New techniques for the grafting of cultured human epidermal cells onto athymic animals. J Invest Dermatol. 1988;91(4):315-8.

80. Driskell RR, Watt FM. Understanding fibroblast heterogeneity in the skin. Trends in cell biology. 2015;25(2):92-9. doi:10.1016/j.tcb.2014.10.001.

81. Collins CA, Kretzschmar K, Watt FM. Reprogramming adult dermis to a neonatal state through epidermal activation of β-catenin. Development (Cambridge, England). 2011;138(23):5189-99. doi:10.1242/dev.064592.

82. Biernaskie J, Paris M, Morozova O, Fagan BM, Marra M, Pevny L et al. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. Cell Stem Cell. 2009;5(6):610-23. doi:10.1016/j.stem.2009.10.019.

83. Rahmani W, Abbasi S, Hagner A, Raharjo E, Kumar R, Hotta A et al. Hair follicle dermal stem cells regenerate the dermal sheath, repopulate the dermal papilla, and modulate hair type. Dev Cell. 2014;31(5):543-58. doi:10.1016/j.devcel.2014.10.022.

84. Festa E, Fretz J, Berry R, Schmidt B, Rodeheffer M, Horowitz M et al. Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell. 2011;146(5):761-71. doi:10.1016/j.cell.2011.07.019.

85. Mecklenburg L, Tobin DJ, Muller-Rover S, Handjiski B, Wendt G, Peters EM et al. Active hair growth (anagen) is associated with angiogenesis. J Invest Dermatol. 2000;114(5):909-16. doi:10.1046/j.1523-1747.2000.00954.x.

86. Yano K, Brown LF, Detmar M. Control of hair growth and follicle size by VEGF-mediated angiogenesis. Journal of Clinical Investigation. 2001;107(4):409-17. doi:10.1172/JCI11317.

87. Stenn KS, Fernandez LA, Tirrell SJ. The angiogenic properties of the rat vibrissa hair follicle associate with the bulb. The Journal of investigative dermatology. 1988;90(3):409-11.

88. Amoh Y, Li L, Yang M, Moossa AR, Katsuoka K, Penman S et al. Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(36):13291-5. doi:10.1073/pnas.0405250101.

89. Xiao Y, Woo WM, Nagao K, Li W, Terunuma A, Mukouyama YS et al. Perivascular hair follicle stem cells associate with a venule annulus. J Invest Dermatol. 2013;133(10):2324-31. doi:10.1038/jid.2013.167.

90. Bigarella CL, Liang R, Ghaffari S. Stem cells and the impact of ROS signaling. Development (Cambridge, England). 2014;141(22):4206-18. doi:10.1242/dev.107086.

91. Hamanaka RB, Glasauer A, Hoover P, Yang S, Blatt H, Mullen AR et al. Mitochondrial reactive oxygen species promote epidermal differentiation and hair follicle development. Science signaling. 2013;6(261):ra8. doi:10.1126/scisignal.2003638.

92. Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell. 2010;7(2):150-61. doi:10.1016/j.stem.2010.07.007.

93. Rathman-Josserand M, Genty G, Lecardonnel J, Chabane S, Cousson A, Francois Michelet J et al. Human hair follicle stem/progenitor cells express hypoxia markers. J Invest Dermatol. 2013;133(8):2094-7. doi:10.1038/jid.2013.113.

94. Chen X, Tian Y, Yao L, Zhang J, Liu Y. Hypoxia stimulates proliferation of rat neural stem cells with influence on the expression of cyclin D1 and c-Jun N-terminal protein kinase signaling pathway in vitro. Neuroscience. 2010;165(3):705-14. doi:10.1016/j.neuroscience.2009.11.007.

95. Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J et al. Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell. 2005;9(5):617-28. doi:10.1016/j.devcel.2005.09.010.

96. Botchkarev VA, Eichmuller S, Johansson O, Paus R. Hair cycle-dependent plasticity of skin and hair follicle innervation in normal murine skin. J Comp Neurol. 1997;386(3):379-95.

97. Brownell I, Guevara E, Bai CB, Loomis CA, Joyner AL. Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell. 2011;8(5):552-65. doi:10.1016/j.stem.2011.02.021.

98. Christoph T, Müller-Röver S, Audring H, Tobin DJ, Hermes B, Cotsarelis G et al. The human hair follicle immune system: cellular composition and immune privilege. The British journal of dermatology. 2000;142(5):862-73.

99. Meyer KC, Klatte JE, Dinh HV, Harries MJ, Reithmayer K, Meyer W et al. Evidence that the bulge region is a site of relative immune privilege in human hair follicles. The British journal of dermatology. 2008;159(5):1077-85. doi:10.1111/j.1365-2133.2008.08818.x.

100. Paus R, Nickoloff BJ, Ito T. A 'hairy' privilege. Trends in immunology. 2005;26(1):32-40. doi:10.1016/j.it.2004.09.014.

101. Osaka N, Takahashi T, Murakami S, Matsuzawa A, Noguchi T, Fujiwara T et al. ASK1-dependent recruitment and activation of macrophages induce hair growth in skin wounds. The Journal of cell biology. 2007;176(7):903-9. doi:10.1083/jcb.200611015.

102. Castellana D, Paus R, Perez-Moreno M. Macrophages contribute to the cyclic activation of adult hair follicle stem cells. PLoS Biol. 2014;12(12):e1002002. doi:10.1371/journal.pbio.1002002.

103. Kloepper JE, Kawai K, Bertolini M, Kanekura T, Paus R. Loss of γδ T Cells Results in Hair Cycling Defects. Journal of Investigative Dermatology. 2013;133(6):1666-9. doi:10.1038/jid.2013.17.

104. Fujiwara H, Ferreira M, Donati G, Marciano DK, Linton JM, Sato Y et al. The basement membrane of hair follicle stem cells is a muscle cell niche. Cell. 2011;144(4):577-89. doi:10.1016/j.cell.2011.01.014.

105. White AC, Khuu JK, Dang CY, Hu J, Tran KV, Liu A et al. Stem cell quiescence acts as a tumour suppressor in squamous tumours. Nat Cell Biol. 2014;16(1):99-107. doi:10.1038/ncb2889.

106. Liu JC, Lerou PH, Lahav G. Stem cells: balancing resistance and sensitivity to DNA damage. Trends in cell biology. 2014;24(5):268-74. doi:10.1016/j.tcb.2014.03.002.

107. Sotiropoulou PA, Candi A, Mascre G, De Clercq S, Youssef KK, Lapouge G et al. Bcl-2 and accelerated DNA repair mediates resistance of hair follicle bulge stem cells to DNA-damage-induced cell death. Nat Cell Biol. 2010;12(6):572-82. doi:10.1038/ncb2059.

108. Rhee H, Polak L, Fuchs E. Lhx2 maintains stem cell character in hair follicles. Science. 2006;312(5782):1946-9. doi:10.1126/science.1128004.

109. Kadaja M, Keyes BE, Lin M, Pasolli HA, Genander M, Polak L et al. SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. Genes Dev. 2014;28(4):328-41. doi:10.1101/gad.233247.113.

110. Folgueras AR, Guo X, Pasolli HA, Stokes N, Polak L, Zheng D et al. Architectural niche organization by LHX2 is linked to hair follicle stem cell function. Cell Stem Cell. 2013;13(3):314-27. doi:10.1016/j.stem.2013.06.018.

111. Vidal VPI, Chaboissier M-C, Lützkendorf S, Cotsarelis G, Mill P, Hui C-C et al. Sox9 Is Essential for Outer Root Sheath Differentiation and the Formation of the Hair Stem Cell Compartment. Current Biology. 2005;15(15):1340-51. doi:10.1016/j.cub.2005.06.064.

112. Nowak JA, Polak L, Pasolli HA, Fuchs E. Hair follicle stem cells are specified and function in early skin morphogenesis. Cell Stem Cell. 2008;3(1):33-43. doi:10.1016/j.stem.2008.05.009.

113. Nishimura EK, Granter SR, Fisher DE. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science. 2005;307(5710):720-4. doi:10.1126/science.1099593.

114. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153(6):1194-217. doi:10.1016/j.cell.2013.05.039.

115. Flores I, Cayuela ML, Blasco MA. Effects of telomerase and telomere length on epidermal stem cell behavior. Science (New York, NY). 2005;309(5738):1253-6. doi:10.1126/science.1115025.

116. Flores I, Blasco MA. The role of telomeres and telomerase in stem cell aging. FEBS letters. 2010;584(17):3826-30. doi:10.1016/j.febslet.2010.07.042.

117. Flores I, Canela A, Vera E, Tejera A, Cotsarelis G, Blasco MA. The longest telomeres: a general signature of adult stem cell compartments. Genes & Development. 2008;22(5):654-67. doi:10.1101/gad.451008.

118. Keyes BE, Segal JP, Heller E, Lien W-H, Chang C-Y, Guo X et al. Nfatc1 orchestrates aging in hair follicle stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(51):E4950-9. doi:10.1073/pnas.1320301110.

119. White AC, Lowry WE. Refining the role for adult stem cells as cancer cells of origin. Trends Cell Biol. 2015;25(1):11-20. doi:10.1016/j.tcb.2014.08.008.

120. Youssef KK, Van Keymeulen A, Lapouge G, Beck B, Michaux C, Achouri Y et al. Identification of the cell lineage at the origin of basal cell carcinoma. Nat Cell Biol. 2010;12(3):299-305. doi:10.1038/ncb2031.

121. Wang GY, Wang J, Mancianti ML, Epstein EH, Jr. Basal cell carcinomas arise from hair follicle stem cells in Ptch1(+/-) mice. Cancer Cell. 2011;19(1):114-24. doi:10.1016/j.ccr.2010.11.007.

122. Kasper M, Jaks V, Are A, Bergstrom A, Schwager A, Svard J et al. Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. Proc Natl Acad Sci U S A. 2011;108(10):4099-104. doi:10.1073/pnas.1014489108.

123. White AC, Tran K, Khuu J, Dang C, Cui Y, Binder SW et al. Defining the origins of Ras/p53-mediated squamous cell carcinoma. Proc Natl Acad Sci U S A. 2011;108(18):7425-30. doi:10.1073/pnas.1012670108.

124. Scheitz CJ, Lee TS, McDermitt DJ, Tumbar T. Defining a tissue stem cell-driven Runx1/Stat3 signalling axis in epithelial cancer. EMBO J. 2012;31(21):4124-39. doi:10.1038/emboj.2012.270.

125. Li S, Park H, Trempus CS, Gordon D, Liu Y, Cotsarelis G et al. A keratin 15 containing stem cell population from the hair follicle contributes to squamous papilloma development in the mouse. Mol Carcinog. 2013;52(10):751-9. doi:10.1002/mc.21896.

# CHAPTER 3

# *GATA6* PROMOTES HAIR FOLLICLE PROGENITOR CELL RENEWAL BY GENOME MAINTENANCE DURING PROLIFERATION [2]

## *3.1 Synopsis*

Cell proliferation is essential to rapid tissue growth and repair, but can result in replication-associated genome damage. Here, we implicate the transcription factor *Gata6* in adult mouse hair follicle regeneration where it controls the renewal of rapidly proliferating epithelial (matrix) progenitors and hence the extent of production of terminally differentiated lineages. We find that *Gata6* protects against DNA damage associated with proliferation, thus preventing cell cycle arrest and apoptosis. Furthermore, we show that *in vivo Gata6* stimulates EDA-receptor signaling adaptor *Edaradd* and NF-κB pathway activation, known to be important for DNA-damage repair and stress response in general, and for hair follicle growth in particular. In cultured keratinocytes, *Edaradd* rescues DNA damage, cell survival and proliferation of *Gata6* knockout cells and restores *Mcm10* expression. Our data add to recent evidence in embryonic stem and neural progenitor cells, suggesting a model whereby

[2] This chapter is adapted from the published article: Wang, A. B., Zhang, Y. V., & Tumbar, T. (2016). *Gata6* promotes hair follicle progenitor cell renewal by genome maintenance during proliferation. *The EMBO Journal*. http://doi.org/10.15252/embj.201694572. YVZ conducted microarray identification of *Gata6* in the hair follicle, qRT–PCR confirmation, colony formation assay, keratinocyte isolation, and contributed to hematoxylin, Gata6, and cH2A.X staining of skin. All other experiments were conducted by ABW. ABW, YVZ, and TT designed the project. ABW and TT wrote the manuscript.

developmentally regulated transcription factors protect from DNA damage associated with proliferation at key stages of rapid tissue growth. Our data may add to understanding why *Gata6* is a frequent target of amplification in cancers.

### *3.2 Introduction*

Many tissue stem cells divide infrequently and are long-lived while their downstream progenitor cells proliferate rapidly and are short-lived (Fuchs & Chen, 2013; Sada & Tumbar, 2013). This hierarchical stem-progenitor system has been presumably set in place to prevent accumulation of replication-associated damage and maintain genome integrity in long-term stem cells (Adams *et al*, 2015). DNA replication-induced stress causes spontaneous double strand breaks in dividing cells, and results in activation of break-repair mechanisms to resume stalled replication forks (Berti & Vindigni, 2016). Hematopoietic stem cells are the best understood, where both cell-intrinsic (*i.e.* transcription factors) and extrinsic (provided by the niche) mechanisms maintain quiescence, to protect the stem cell genome in long term against DNA damage (Kosan & Godmann, 2016) and prevent aging (Flach *et al*, 2014). This depends on full replication-origin licensing via MCM3 (Alvarez *et al*, 2015). In the hair follicle, a mutation that promotes proliferation of otherwise quiescent hair follicle stem cells (HFSCs) also leads to increased DNA damage and premature aging (Morgner *et al*, 2015).

Some tissue stem cells *in vivo* (*i.e.* epidermis) and pluripotent stem cells in culture divide relatively frequently and mechanisms other than quiescence may protect against replication-induced stress (Sotiropoulou *et al*, 2012; Weissbein *et al*, 2014). Similarly, some rapidly dividing progenitor cells must provide essential tissue

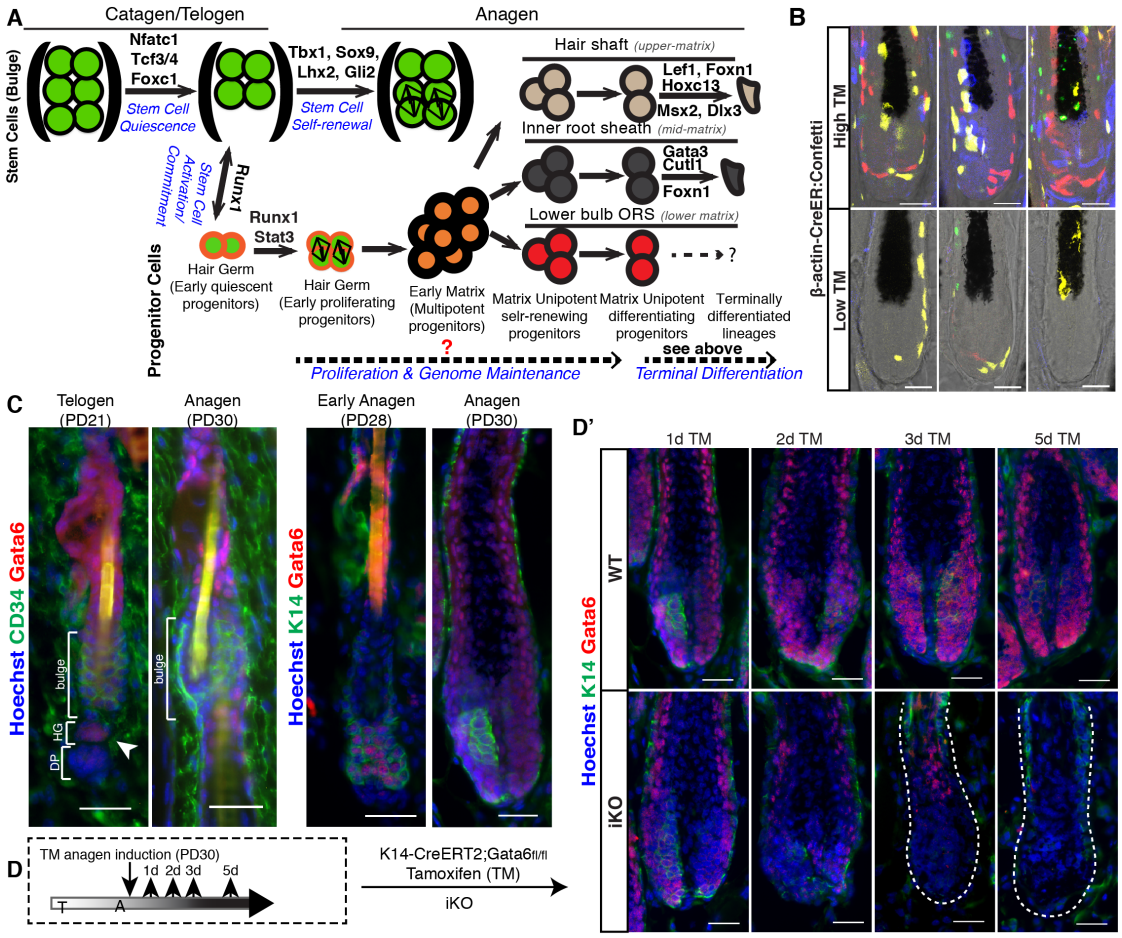
regenerative capacity for substantial periods of time. Developmental mechanisms may be set in place in a cell-type specific manner to prevent catastrophic accumulation of DNA damage during times of intense tissue growth and repair. Recently, two developmental transcription factors , *SRF* in pluripotent stem cells (Lamm *et al*, 2016) and *MYCN* in neural progenitors (Petroni *et al*, 2016), were shown to control replication-induced stress in rapidly proliferating cells. However, the extent to which developmentally-regulated pathways or transcription factors may be implicated in augmenting the ability of highly proliferative cells to maintain genomic integrity during replication at key stages in tissue growth is currently unknown.

The hair follicle is a classical stem-progenitor model system and is particularly well-suited for studying mechanisms of genome integrity related to differences in cell proliferative behavior during adult homeostasis. The HFSCs are infrequently dividing and long-lived and are localized in the bulge, whereas the progenitor cells are rapidly dividing, have a short life span, and are localized in the matrix within the hair bulb. Additionally, hairs undergo cyclic and synchronous, at least during youth, phases of quiescence (telogen), growth (anagen), and destruction (catagen), known as the hair cycle. Proliferation of stem and progenitor cells are restricted within highly defined stages of the hair cycle. At the start of anagen, quiescent early progenitor cells in the hair germ begin to proliferate to form a pool of multipotent progenitors known as the matrix. Matrix progenitor cells rapidly proliferate, temporarily self-renew, and eventually become lineage restricted unipotent progenitors with the hair shaft (HS) committed cells localized in the upper matrix, the inner root sheath (IRS) in the mid-matrix, and the lower bulb ORS in the lower matrix (Legué & Nicolas, 2005; Legué *et*

*al*, 2010). The unipotent progenitor cells divide briefly when displaced from the dermal papillae (DP), a mesenchymal pocket of cells working as an organizing and signaling center in the hair follicle, and then withdraw from the hair cycle and terminally differentiate to IRS and HS lineages (Legué & Nicolas, 2005; Legué *et al*, 2010).

Defined steps in the stem cell lineage determination hierarchy of HFSCs have been intensively studied and their regulation by known developmental transcription factors is summarized in Figure 3.1A. Specifically, *NFATc1*, *Tcf3/4*, and *Foxc1* are important for maintaining bulge stem cell quiescence (Horsley *et al*, 2008; Wang *et al*, 2016; Lien *et al*, 2014; Lay *et al*, 2016), while *Tbx1*, *Sox9*, *Lhx2*, and *Gli2* are known to promote bulge cell self-renewal (Chen *et al*, 2012; Kadaja *et al*, 2014; Folgueras *et al*, 2013; Hsu *et al*, 2014). *Runx1* promotes initial activation of bulge stem cells during quiescence to become hair germ cells that are subsequently capable of conducting timely anagen onset (Lee *et al*, 2014; Osorio *et al*, 2008; 2011; Hoi *et al*, 2010; Lee *et al*, 2013). *Stat3* also plays a role in anagen onset by regulating proliferation of hair germ cells (Sano *et al*, 1999; 2000). During terminal differentiation of matrix progenitor cells, *Dlx3*, *Foxn1*, *Msx2*, *Hoxc13*, and *Lef1* are known to promote differentiation towards the hair shaft lineage (Hwang *et al*, 2008; Weiner *et al*, 2007; Hu *et al*, 2010a; Merrill *et al*, 2001; Lowry *et al*, 2005; Ma *et al*, 2003; Kim & Yoon, 2013; Jave-Suarez *et al*, 2002; DasGupta & Fuchs, 1999; Zhou *et al*, 1995), while *Cutl1*, *Gata3*, and *Foxn1* promote differentiation towards the IRS lineage (Ellis *et al*, 2001; Kaufman *et al*, 2003; Cai *et al*, 2009).

**Figure 3.1** Hair follicle progenitor cells express *Gata6*. (**A)** Overview of hair follicle stem and progenitor cell dynamics with transcription factors known to regulate each stage. (**B**) Lineage tracing of the anagen matrix from *β-actin-CreER:Confetti*, 7 days after induction with either a high dose of tamoxifen (TM) (top) or low dose (bottom) for single cell clonal lineages. (**C**) Images of skin sections at various stages of the hair cycle, immunostained for GATA6 (red), CD34 or K14 (green) and DNA (blue). (**D**) TM induction scheme (D) and images (D') for *Gata6* inducible knockout (iKO) and wild type (WT) mice over time, showing the loss of GATA6 (red) from the hair follicle. Scale bars: 30 μm.


A
Catagen/Telogen
Anagen
Stem Cells (Bulge)
Nfatc1
Tcf3/4
Foxc1
Stem Cell
Quiescence
Tbx1, Sox9,
Lhx2, Gli2
Stem Cell
Self-renewal
Hair shaft (upper-matrix)
Lef1, Foxn1
Hoxc13
Msx2, Dlx3
Inner root sheath (mid-matrix)
Gata3
Cutl1
Foxn1
Lower bulb ORS (lower matrix)
Stem Cell
Activation/
Commitment
Runx1
Runx1
Stat3
Progenitor Cells
Hair Germ
(Early quiescent
progenitors)
Hair Germ
(Early proliferating
progenitors)
Early Matrix
(Multipotent
progenitors)
Matrix Unipotent
selr-renewing
progenitors
Matrix Unipotent
differentiating
progenitors
Terminally
differentiated
lineages
see above
?
Proliferation & Genome Maintenance
Terminal Differentiation
B
β-actin-CreER:Confetti
High TM
Low TM
C
Telogen
(PD21)
Anagen
(PD30)
Early Anagen
(PD28)
Anagen
(PD30)
Hoechst CD34 Gata6
bulge
HG
DP
Hoechst K14 Gata6
D
TM anagen induction (PD30)
1d 2d 3d 5d
T
A
K14-CreERT2;Gata6fl/fl
Tamoxifen (TM)
iKO
D'
1d TM
2d TM
3d TM
5d TM
WT
iKO
Hoechst K14 Gata6

The developmental transcription factors regulating intermediate steps in hair follicle lineage determination, specifically matrix progenitor cell proliferation, extent of self-renewal, and genome maintenance of these rapidly proliferative cells are currently not known (Fig 3.1A). With that said, components of DNA repair machinery, namely *p53* and *BRCA1* were shown to be crucial in the hair follicle matrix cells (but not in the inter-follicular epidermal cells) for their genome maintenance during normal homeostasis, suggesting the existence of significant genotoxic stress in the progenitor matrix cells (Sotiropoulou *et al*, 2012). Furthermore, during anagen, Sonic hedgehog (Shh) and Wnt signaling are implicated in matrix cell proliferation (Oro & Higgins, 2003; St-Jacques *et al*, 1998; Wang *et al*, 2000; Hsu *et al*, 2014; Choi *et al*, 2013).

Here we set to identify transcription factors controlling progenitor matrix cell function and implicate the zinc-finger factor *Gata6*, in the activation and extent of renewal of the hair follicle progenitor cells (hair germ and matrix). *Gata6* was previously known to regulate development of visceral endoderm, liver, lung, heart, and intestine (Morrisey *et al*, 1998; Zhao *et al*, 2005; Yang *et al*, 2002; Zhang *et al*, 2008; Beuling *et al*, 2011; 2012; Zhao *et al*, 2008) through control of differentiation, survival and proliferation, but a role in DNA damage has not yet been reported to our knowledge. Our new findings from the hair follicle implicate *Gata6* in prevention of DNA-damage associated with proliferation, and may refine our understanding of *Gata6* phenotypes in other tissue stem-progenitor cell systems (Zhang *et al*, 2008), as well as provide an important link with its known role as an oncogene (Kwei *et al*, 2008; Shureiqi *et al*, 2007; Lin *et al*, 2012; Shen *et al*, 2013; Whissell *et al*, 2014).

Moreover, we expand *Gata6* function, previously thought restricted to the mesoderm- and endoderm-derived lineages (Molkentin, 2000; Maeda *et al*, 2005), into an ectodermal tissue.

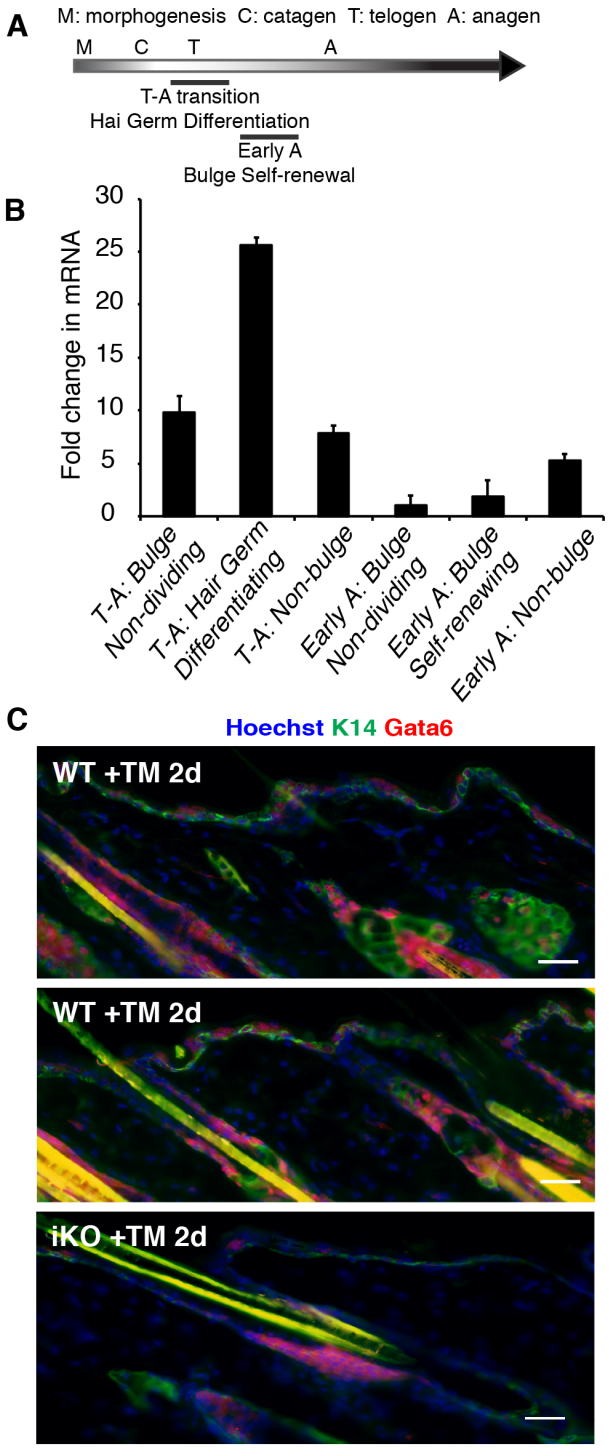
### *3.3 Gata6 is expressed in hair follicle progenitor cells*

Previously, lineage tracing experiments of early progenitor (hair germ) and progenitor (matrix) cells have shown that multipotent early progenitors become lineage restricted within the matrix to form either the inner root sheath or hair shaft, while it was suggested that a pool of cells at the base of the matrix may contribute to the lower outer root sheath in the hair bulb (Zhang *et al*, 2009; Legué & Nicolas, 2005; Legué *et al*, 2010). Furthermore, it has been proposed that the matrix is structured such that lineage specification correlates with position of cells along the length of the matrix, and proliferative potential correlates with radial position relative to the DP (Legué & Nicolas, 2005). Using the ubiquitously expressed *β-actin-CreER* mice and a multi-color reporter not previously employed to study matrix-cell differentiation (*Confetti* reporter mice) (Snippert *et al*, 2010), we performed our own lineage tracing during anagen by inducing with tamoxifen (TM) followed by 4 day chases. High TM doses (100ug/g body) induced multi-color labeling within one lineage emanating from multiple progenitors contacting the DP at specific vertical positions that contribute simultaneously to hair growth. Low TM doses (20ug/g body) produced single color-single lineage patterns (Fig 3.1B), consistent with the previously proposed model (Legué & Nicolas, 2005).

Next we set out to identify transcription factors that may regulate matrix progenitor cell function. Previously, we performed microarray analysis of HFSC lineages at distinct stages of self-renewal (anagen) and early differentiation (telogen-anagen transition) (Zhang *et al*, 2009). These data revealed *Gata6* as a transcription factor that was up-regulated in dividing hair germ cells on their way to forming matrix but not in dividing bulge cells that were self-renewing at later anagen stages. We confirmed the microarray data here by qRT-PCR of sorted hair follicle cell populations (Fig 3.2A,B). These data suggested that *Gata6* might play a specific role in the proliferation of hair germ (but not bulge) cells during matrix progenitor cell formation and prompted us to further study its expression and role in the hair follicle.

We evaluated GATA6 protein expression patterns during the hair cycle by immunofluorescence staining. Consistent with our microarray data, immunofluorescence staining revealed GATA6 expression in the hair germ but not bulge during telogen and early anagen (Fig 3.1C). Expression was high throughout the entire matrix at anagen, including all matrix zones represented by progenitors of lower ORS, IRS and cortex, and remained high during full and late anagen (Fig 3.1C and D – top panel). Moreover, expression was high in the differentiated hair layers of inner root sheath (Fig 3.1C, D). GATA6 was also present in the infundibulum (the upper portion of the hair follicle) and in the inter-follicular epidermis (Figs 3.1C and 3.2C). In the bulge, GATA6 was detectable in rare cells in the differentiating zone bordering the hair germ during early anagen, but was absent from the bulge during later stages of HFSCs self-renewal (early and mid-anagen) (Fig 3.1C). These data suggested that *Gata6* might play a role in matrix cell formation, proliferation, and differentiation.

**Figure 3.2** *Gata6* is upregulated in differentiating hair germ cells. (**A**) Experimental scheme for doxycycline chased *K5tTA x pTRE-H2B-GFP* mice used to obtain divided and un-divided cell populations during the differentiating phase - telogen (T)-anagen (A) transition - and self-renewing phase of HFSCs during early anagen, as previously described in (Zhang *et al*, 2009). Bulge cells were sorted as divided or un-divided based on H2B-GFP levels and high CD34 and α6-integrin expression; hair germ cells at telogen-anagen transition were the divided cells with low CD34 levels and high expression of α6-integrin. (**B**) qRT-PCR confirmation of Gata6 expression in differentiating (hair germ, divided), self-renewing (bulge, divided), or non-dividing bulge stem cells (average ± SD, n=3). (**C**) Immunofluorescence image of the skin shows epidermis and infundibulum stained with Hoechst for DNA (blue) basal layer/ORS marker K14 (green) and Gata6 (red). Scale bar: 30 μm. (**D**) Gel image of DNA genotyped for floxed Gata6 allele. Shown are genotypes from 3 mice: heterozygote ($Gata6^{fl/wt}$), homozygous WT ($Gata6^{wt/wt}$), and homozygous floxed ($Gata6^{fl/fl}$).


A
M: morphogenesis C: catagen T: telogen A: anagen
M C T A
T-A transition
Hai Germ Differentiation
Early A
Bulge Self-renewal
B
Fold change in mRNA
30
25
20
15
10
5
0
T-A: Bulge Non-dividing
T-A: Hair Germ Differentiating
T-A: Non-bulge
Early A: Bulge Non-dividing
Early A: Bulge Self-renewing
Early A: Non-bulge
C
Hoechst K14 Gata6
WT +TM 2d
WT +TM 2d
iKO +TM 2d
D
Gata6: fl/wt wt/wt fl/fl
250 bp fl
159 bp wt

### *3.4 Loss of Gata6 causes hair follicle degeneration*

To investigate the role of *Gata6* in the hair follicle, we performed inducible knockout of *Gata6* in the skin epithelium of *K14-CreER$^{T2}$;Gata6$^{fl/fl}$* mice (*Gata6* iKO) (Li *et al*, 2000; Sodhi *et al*, 2006). Upon induction with TM, GATA6 is rapidly lost from the lower hair follicle (Fig 3.1D) and epidermis (Fig 3.2C). Control littermate mice without TM induction (*K14-CreER$^{T2}$;Gata6$^{fl/fl}$* injected with oil), without *Cre* expression (*Gata6$^{fl/fl}$* injected with TM), or without *Gata6* loxP sites (*K14-CreER$^{T2}$* injected with TM) showed no phenotypic effects and are referred to as wild type (WT) throughout the paper. A time course of immunofluorescence staining following induction in anagen shows that GATA6 is initially lost from matrix cells bordering the DP, followed by progressive loss from the rest of the matrix within 2 days post-TM, and finally from the differentiated lineages by 5 days after induction (Fig 3.1D). Given the presence of GATA6 in both the telogen/early anagen hair germ and the anagen matrix, we asked how loss of *Gata6* affects the hair cycle at these two stages (Fig 3.3A). When *Gata6* loss is induced at telogen, hair follicles are arrested in telogen in iKO mice while WT littermates progress into anagen by 10 days (Fig 3.3B and 3.4A). This was true in all hair follicles from iKO and WT mice tested ($n \geq 3$ mice/genotype) in multiple experiments with skin sections from different parts of the body. Not only do telogen induced iKO follicles fail to enter anagen, the hair germ of telogen iKO follicles shrinks in size suggesting loss of the early progenitor cells in the absence of *Gata6*. A more dramatic reduction in hair follicle progenitor cells occurs when GATA6 is depleted during anagen, when matrix progenitor cells are rapidly

**Figure 3.3** *Gata6* is necessary for anagen initiation and maintenance. (**A**) Induction scheme for generating the *Gata6* iKO. Mice were induced during either telogen (PD21) or anagen (PD30) for up to 10 days ($n \geq 3$ mice/time point/genopye). (**B**) Representative images of hematoxylin staining of skin sections from the telogen induced mice sacrificed at 2, 4, and 10 days (d) post TM induction. (**C**) Images of hematoxylin staining of skin section from the anagen induced mice. Quantification shown in Figure 3.4D. Scale bars: 30 μm.

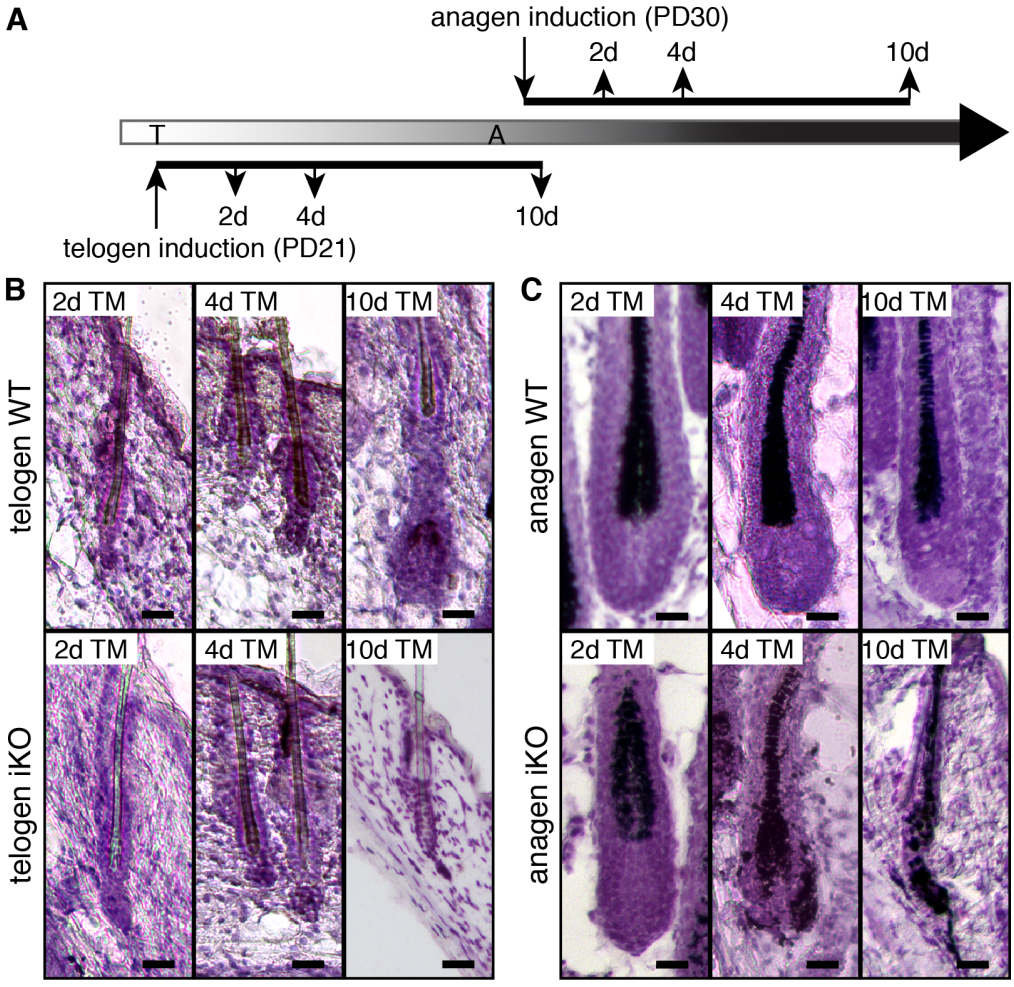
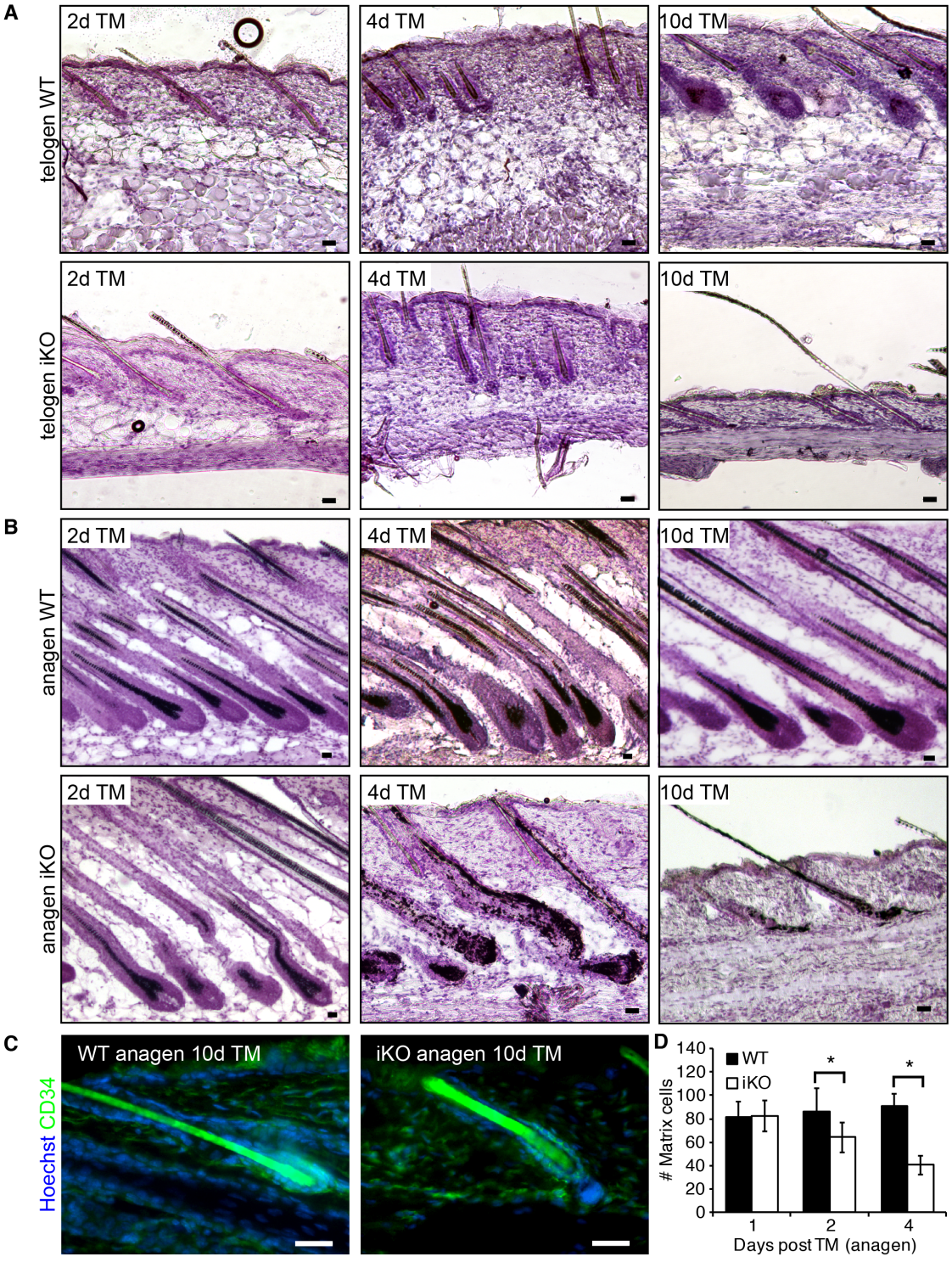

A
anagen induction (PD30)
2d
4d
10d
T
A
2d
4d
10d
telogen induction (PD21)
B
2d TM
4d TM
10d TM
telogen WT
2d TM
4d TM
10d TM
telogen iKO
C
2d TM
4d TM
10d TM
anagen WT
2d TM
4d TM
10d TM
anagen iKO

**Figure 3.4** *Gata6* is necessary for anagen initiation and maintenance. (**A-B**) Low magnification images of skin with hematoxylin staining, for telogen induced (**A**) and anagen induced (**B**) mice. Scale bars: 30 μm. (**C**) Immunofluorescence images of hair follicles 10 days post anagen induction stained with bulge marker CD34. (**D**) Quantification of the number of cells in anagen matrix hair follicles post *Gata6* iKO induction (average ± SD, n=20 follicles from 3 mice each). *Unpaired t-test P-values: 1d = 0.86, 2d = $7x10^{-5}$, 4d = $4x10^{-10}$.


A
telogen WT
2d TM
4d TM
10d TM
telogen iKO
2d TM
4d TM
10d TM
B
anagen WT
2d TM
4d TM
10d TM
anagen iKO
2d TM
4d TM
10d TM
C
Hoechst CD34
WT anagen 10d TM
iKO anagen 10d TM
D
# Matrix cells
140
120
100
80
60
40
20
0
WT
iKO
*
*
1
2
4
Days post TM (anagen)

proliferating and differentiating. Over the course of 10 days, WT follicles progress through full anagen. In contrast, anagen iKO follicles display reduced matrix size, accumulate ectopic melanin granules, and by 10 days degenerate to a telogen like structure (Figs 3.1D, 3.3C and 3.4B,D) in all mice analyzed ($n \geq 3$ mice/genotype/time point). Consistent with our observed localization of GATA6 within the matrix and its absence in the bulge, these changes to the hair structure appear to be limited to the lower hair follicle, while the bulge morphology remains intact during these stages (Fig 3.4C). Also, no gross phenotype was detectable in the epidermis.

Together, these data demonstrate that *Gata6* is necessary for the telogen-anagen transition and hair germ proliferation and maintenance. In addition, it is absolutely required to maintain the hair follicle matrix and normal hair growth during anagen and to prevent its premature degeneration with return to telogen.

### *3.5 Gata6 is not necessary for hair follicle progenitor cell terminal differentiation*

Previously, *Gata6* has been implicated in the differentiation of lung epithelial progenitor cells (Zhang *et al*, 2008). To determine if loss of *Gata6* in the hair follicle progenitors (matrix) during anagen plays a role in terminal differentiation, we performed immunofluorescence staining of skin sections with antibodies including markers for the differentiated lineages of the hair. The stainings included the ORS marker K14, the IRS and medulla marker AE15, the hair cortex marker AE13, GATA3 that marks the cuticle and Huxley layers of the IRS, the companion layer marker K6, and hair shaft cortex marker LEF1. Surprisingly, the differentiated hair lineages were detectable in *Gata6* iKO not only at 2 days but also at 5 days after TM

induction (Fig 3.5 and 3.6A-D), when GATA6 expression was completely lost from the hair follicle as indicated by our staining (Fig 3.1D). This suggested that *Gata6* is not directly implicated in terminal differentiation of hair follicle cells, as expected from its role in other systems.

To more directly inquire if Gata6 iKO matrix cells actually continue to proliferate, produce terminally differentiated cells, and move upward into the inner hair follicle lineages above the DP after GATA6 loss, we conducted a BrdU pulse-chase experiment to label the matrix cells after TM induction and follow their fate. Mice were injected with BrdU three days after tamoxifen induction, when GATA6 is normally lost in the matrix (Fig 3.1D) and sacrificed at 12 hrs and at 3 days after injection by which time GATA6 is also lost from the differentiated lineages (Fig 3.5A). As expected, after 12 hours of chase, the BrdU labeling is predominantly located in the matrix and this was true in both WT and iKO mice (Fig 3.5B and 3.6E). As the matrix cells differentiate, they feed upwards into the IRS and hair shaft. Indeed, after 2 days chase the BrdU label appeared in the zone occupied by terminally differentiated lineages above the DP in both WT and iKO skin, where they co-localize with differentiation markers AE15 and AE13 (Fig 3.5D,E and 3.6E). Additionally, the 5-day *Gata6* iKO follicles also express all other differentiation markers tested, namely GATA3, K6, and LEF1 in a manner similar to that detected in WT hair follicles (Fig 3.5F,G).

These data showed that despite the rapid shrinkage of matrix and loss of hair follicle growth with premature collapse into early catagen in response to *Gata6* iKO,

**Figure 3.5** *Gata6* is not necessary for terminal differentiation of matrix cells. (**A**) Scheme for BrdU pulse chase experiment to track matrix cell differentiation into the upper inner lineages during anagen. The plus sign marks the starting point for TM or BrdU. (**B-G**) *Gata6* WT and iKO skin after 3 days after TM induction and 12 hours after BrdU labeling or 5 days after TM induction and 2 days after BrdU labeling stained with markers as indicated in corresponding color.

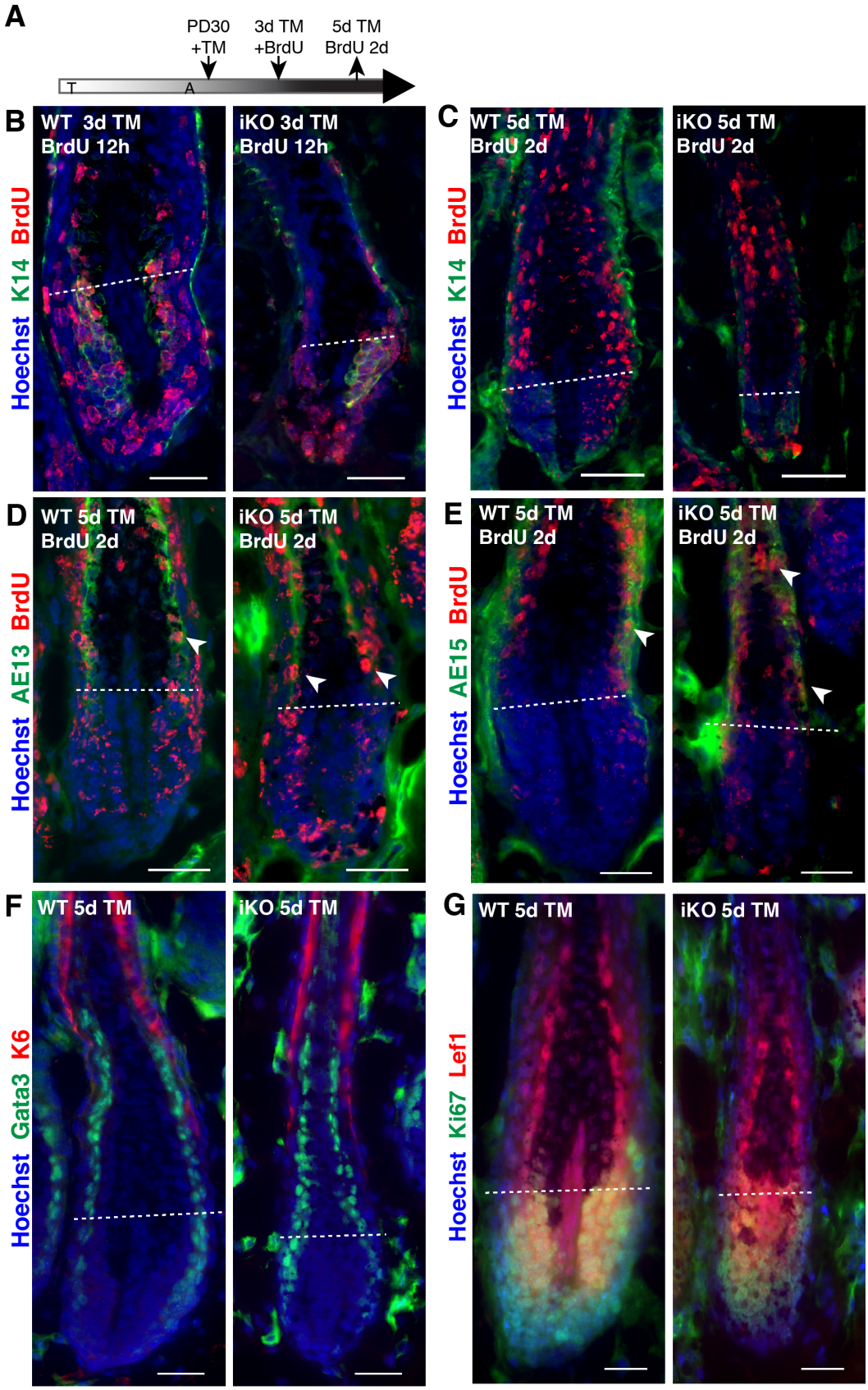
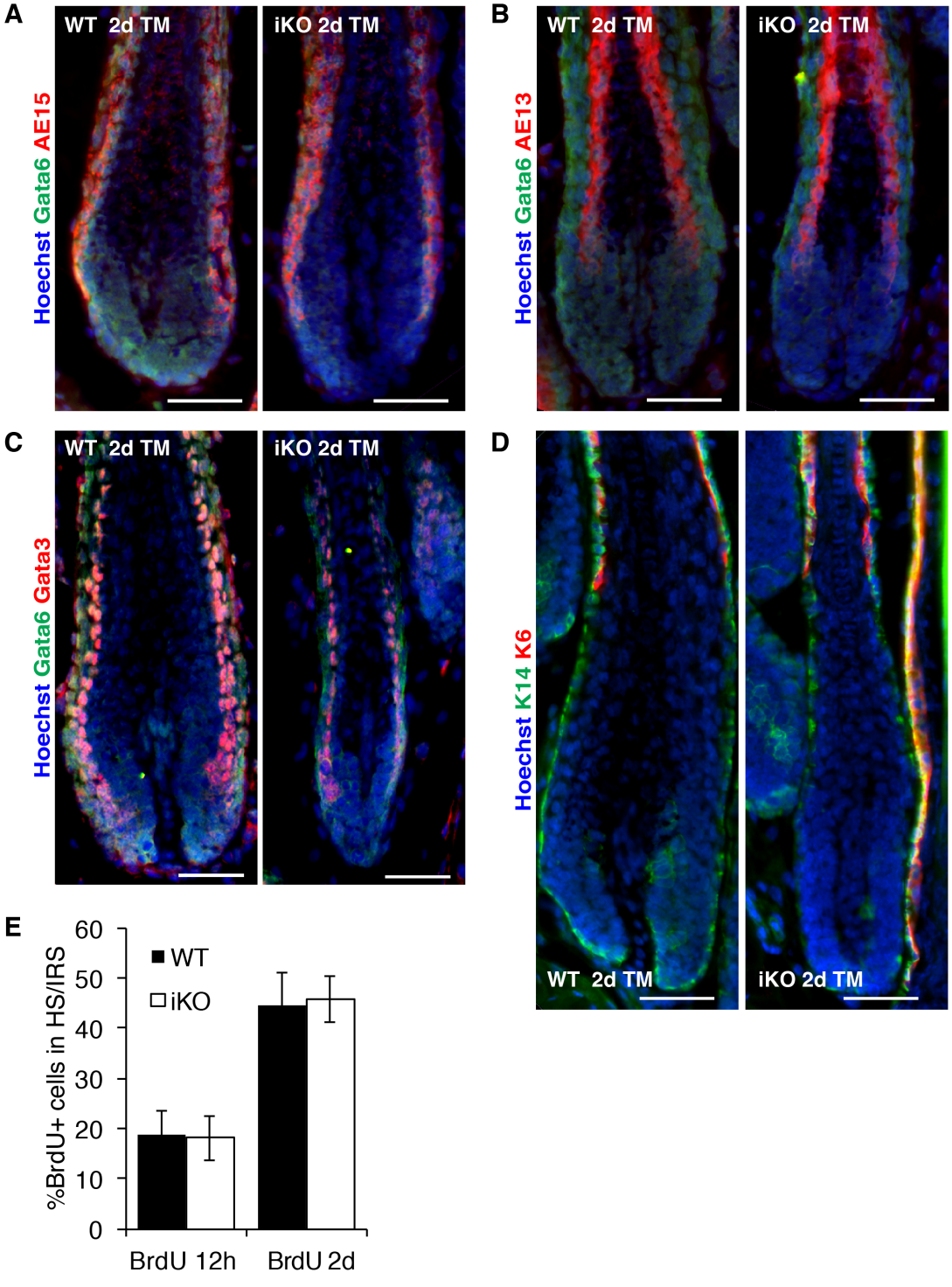

A
PD30
+TM
3d TM
+BrdU
5d TM
BrdU 2d
T
A
B
WT 3d TM
BrdU 12h
iKO 3d TM
BrdU 12h
Hoechst K14 BrdU
C
WT 5d TM
BrdU 2d
iKO 5d TM
BrdU 2d
Hoechst K14 BrdU
D
WT 5d TM
BrdU 2d
iKO 5d TM
BrdU 2d
Hoechst AE13 BrdU
E
WT 5d TM
BrdU 2d
iKO 5d TM
BrdU 2d
Hoechst AE15 BrdU
F
WT 5d TM
iKO 5d TM
Hoechst Gata3 K6
G
WT 5d TM
iKO 5d TM
Hoechst Ki67 Lef1

**Figure 3.6** *Gata6* is not necessary for terminal differentiation of the hair follicle. (**A-D**) Immunofluorescence staining of skin sections 2 days post tamoxifen induction for terminally differentiated lineages reveal labeling in the hair bulb, as expected. Specific stains are indicted in corresponding colors. Scale bars: 30 μm. (**E**) Quantification of images like those shown in Figure 3 show percentage of BrdU+ cells located in the differentiated IRS and hair shaft lineages after the initial 12h pulse labeling and after the 2d BrdU chase (average ± SD, n=3, P-value = 0.7 for 12h WT vs iKO and 0.4 for 2d WT vs iKO).

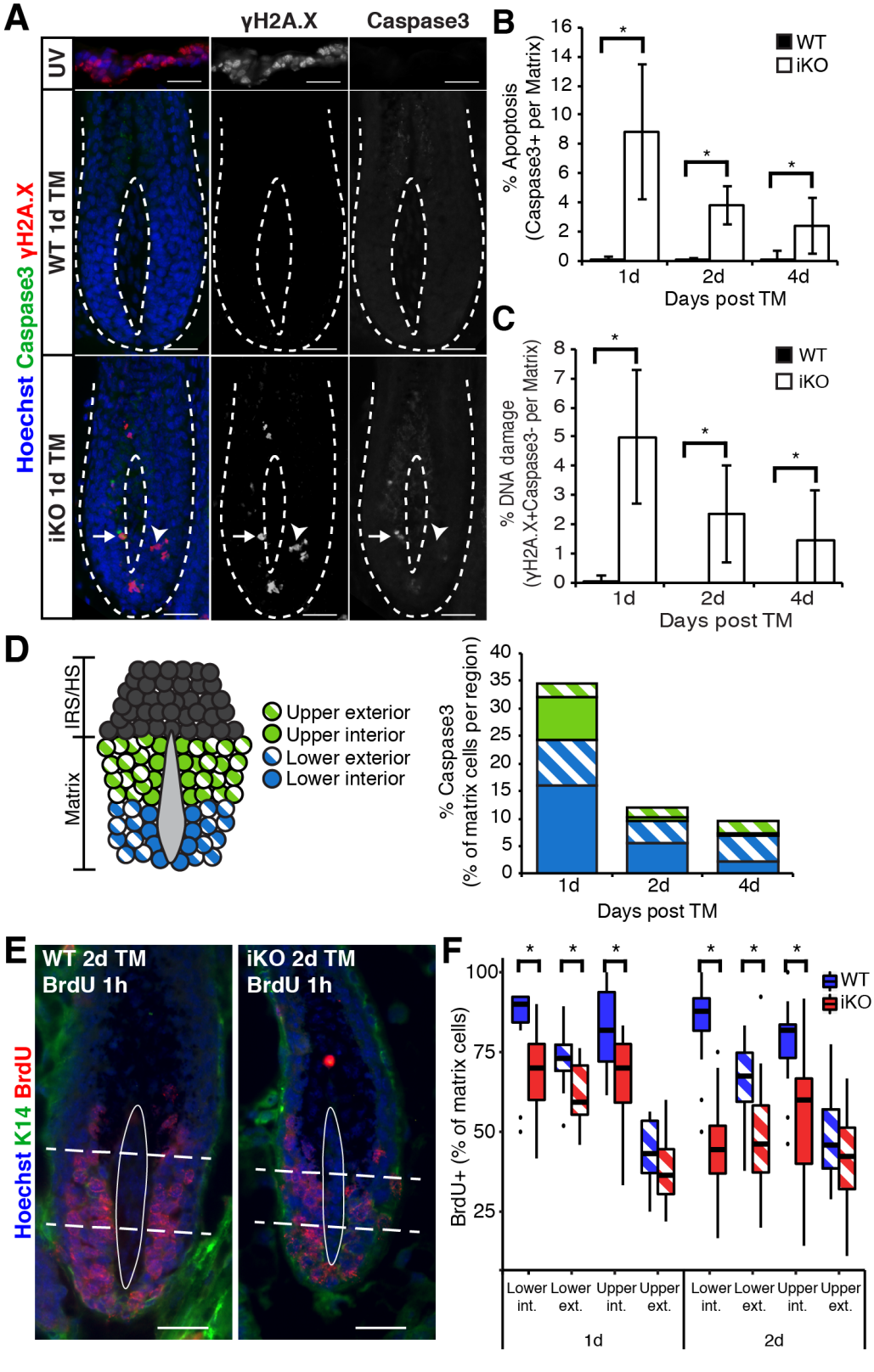

A
WT 2d TM
iKO 2d TM
Hoechst Gata6 AE15
B
WT 2d TM
iKO 2d TM
Hoechst Gata6 AE13
C
WT 2d TM
iKO 2d TM
Hoechst Gata6 Gata3
D
Hoechst K14 K6
WT 2d TM
iKO 2d TM
E
%BrdU+ cells in HS/IRS
60
50
40
30
20
10
0
WT
iKO
BrdU 12h
BrdU 2d

hair follicle matrix progenitors continue to produce all the differentiated inner layer lineages, suggesting that *Gata6* is not essential for commitment to terminal differentiation. Thus, we set to examine whether there was a potential impairment in the extent of matrix cell proliferation or survival during anagen, which would explain the abnormal shrinkage of the iKO matrix.

### *3.6 Matrix cells undergo DNA damage, apoptosis, and reduced proliferation after loss of Gata6*

The rapid degeneration of the hair follicle and ectopic melanin granules observed after loss of GATA6 were reminiscent of the dystrophic catagen morphology frequently observed in response to severe damage, such as following chemotherapy (Hendrix *et al*, 2005). We therefore analyzed whether genomic damage and cell death are observed in *Gata6* iKO matrix cells by immunofluorescence staining of skin sections from mice sacrificed at different time-points after TM injection. We used antibodies for the DNA damage marker γH2A.X, which localizes to double-strand breaks, and for the apoptosis marker activated Caspase 3 (Fig 3.7A). *Gata6* iKO hair follicles displayed frequent matrix cells that were either Caspase3+/γH2A.X+ (indicative of cell death) or Caspase3-/γH2A.X+ (indicative of DNA damage), while in WT matrix these cells were largely absent. Quantification of these data demonstrated that the number of cells with DNA damage and/or apoptosis peaked at 1-day post-TM induction, and was followed by a gradual decline by 4 days post-TM (Fig 3.7B,C). These data suggested that GATA6 loss results in drastic DNA damage

**Figure 3.7** Hair follicle progenitor show impaired proliferation and increased DNA damage and apoptosis following loss of *Gata6*. (**A**) Hair follicles exhibit immunostaining signal for DNA damage marker γH2A.X (red) and apoptosis marker Caspase3 (green) as early as one day after Gata6 iKO induction. Top panel shows a section of UV irradiated epidermis as a positive control for DNA damage. Note that Caspase 3 is not expressed in the γH2A.X cells of the epidermis but is found in some iKO hair follicle matrix cells. Right panels show single color images as indicated at top (**B**) Quantification (average ± SD) of the percentage of apoptotic cells per matrix as determined by counting of Caspase3+ cells (n=50 follicles from 3 mice per group) (**C**) Quantification (average ± SD) of the percentage of DNA damaged cells per matrix as determined by counting of γH2A.X+Caspase3- cells (n=50 follicles from 3 mice per group). (**D**) Regional distribution of apoptosis was analyzed by dividing the matrix into four compartments as shown in the legend (left) and counting the fraction of Caspase3+ cells per each matrix compartments (right; n=50 follicles from 3 mice per group). (**E**) Proliferation in the matrix compartments shown in (D) was assessed by BrdU labeling for 1 hour, followed by staining, and quantification (**F**) (interior cells labeled with solid bars, exterior cells labeled with striped bars). Unpaired t-test: *P-value < 0.05. n=20 follicles from 2 mice per group.


A
γH2A.X
Caspase3
UV
WT 1d TM
iKO 1d TM
Hoechst Caspase3 γH2A.X
B
% Apoptosis (Caspase3+ per Matrix)
WT
iKO
1d
2d
4d
Days post TM
C
% DNA damage (γH2A.X+Caspase3- per Matrix)
WT
iKO
1d
2d
4d
Days post TM
D
IRS/HS
Matrix
Upper exterior
Upper interior
Lower exterior
Lower interior
% Caspase3 (% of matrix cells per region)
1d
2d
4d
Days post TM
E
WT 2d TM
BrdU 1h
iKO 2d TM
BrdU 1h
Hoechst K14 BrdU
F
BrdU+ (% of matrix cells)
WT
iKO
Lower int.
Lower ext.
Upper int.
Upper ext.
1d
2d

followed by apoptosis in the matrix cells and were in line with the observed shrinking of the matrix cells over the same time period (Fig 3.1D). Neither DNA damage nor apoptosis was found within the bulge stem cells (Fig 3.8); this was expected since these cells do not express GATA6 during anagen (Fig 3.1C). Additionally, although the inter-follicular epidermis and infundibulum express GATA6 (Fig 3.1C and 3.2C), neither DNA damage nor apoptosis was observable (Fig 3.8), perhaps due to the action of additional DNA-damage protective mechanisms that seem to operate in these compartments (Sotiropoulou *et al*, 2012).

Since the different unipotent matrix progenitors are spatially compartmentalized along the proximal-distal and lateral axis (Legué & Nicolas, 2005), we quantified the distribution of Caspase3 staining in different regions of the matrix (Fig 3.7D). The matrix was divided vertically into a lower region from the base of the matrix to the middle of the DP, and an upper region from the middle of the DP to the Line of Auber, where the IRS and cortex of the hair shaft begin. Additionally, along the radial axis, we divided the matrix into an interior region that is adjacent to the DP, and the exterior region that does not contact the DP. The immediate vicinity to the DP has been deemed essential for the extent of self-renewal of unipotent progenitor matrix cells (Legué & Nicolas, 2005). Careful quantification of the frequency of Caspase3+ cells per region demonstrated that all matrix cells were affected by apoptosis, irrespective of their spatial distribution. Although there were some differences in frequency of apoptotic cells in the different categories, these were likely a reflection of how rapidly GATA6 loss occurred in distinct matrix regions as the cells near the DP lose GATA6 first, immediately after induction (Fig 3.1D).

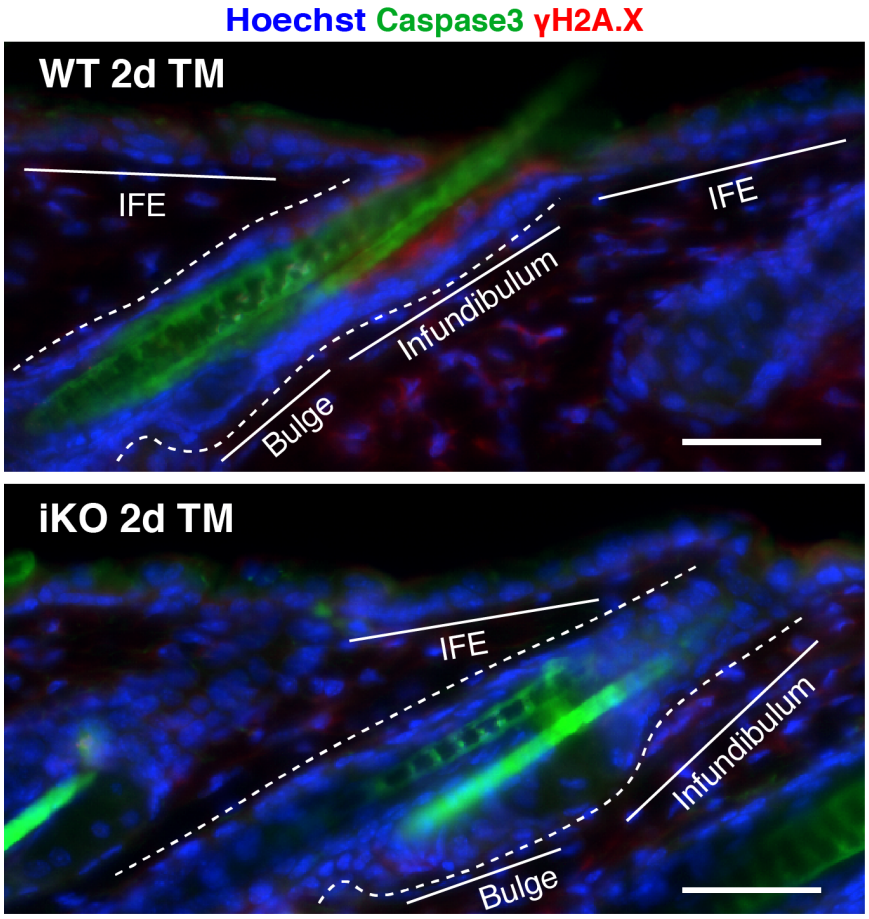



**Figure 3.8** *Gata6* loss does not cause DNA damage or apoptosis in the interfollicular epidermis or bulge. Skin sections immunostained for Caspase3 and γH2A.X show no detectable signal in the inter-follicular epidermis or bulge HFSCs.

We also investigated the effect of *Gata6* loss on proliferation in different regions of the matrix in WT and iKO mice injected with BrdU 1 hour before sacrifice (Fig 3.7E,F). As expected based on the vicinity from the DP (Legué & Nicolas, 2005), the WT matrix displayed more BrdU+ cells in the inner versus the outer regions. The upper exterior matrix region, is the least proliferative, as it includes terminally differentiated cells of the IRS, and *Gata6* loss had a subtle effect in this region by 2 days post-TM. However, significant decrease in the fraction of BrdU+ cells was detectable in all other examined matrix categories within 1 and 2 days of TM injection (Fig 3.7E,F), suggesting *Gata6* function is essential for normal proliferation of all matrix progenitor cells.

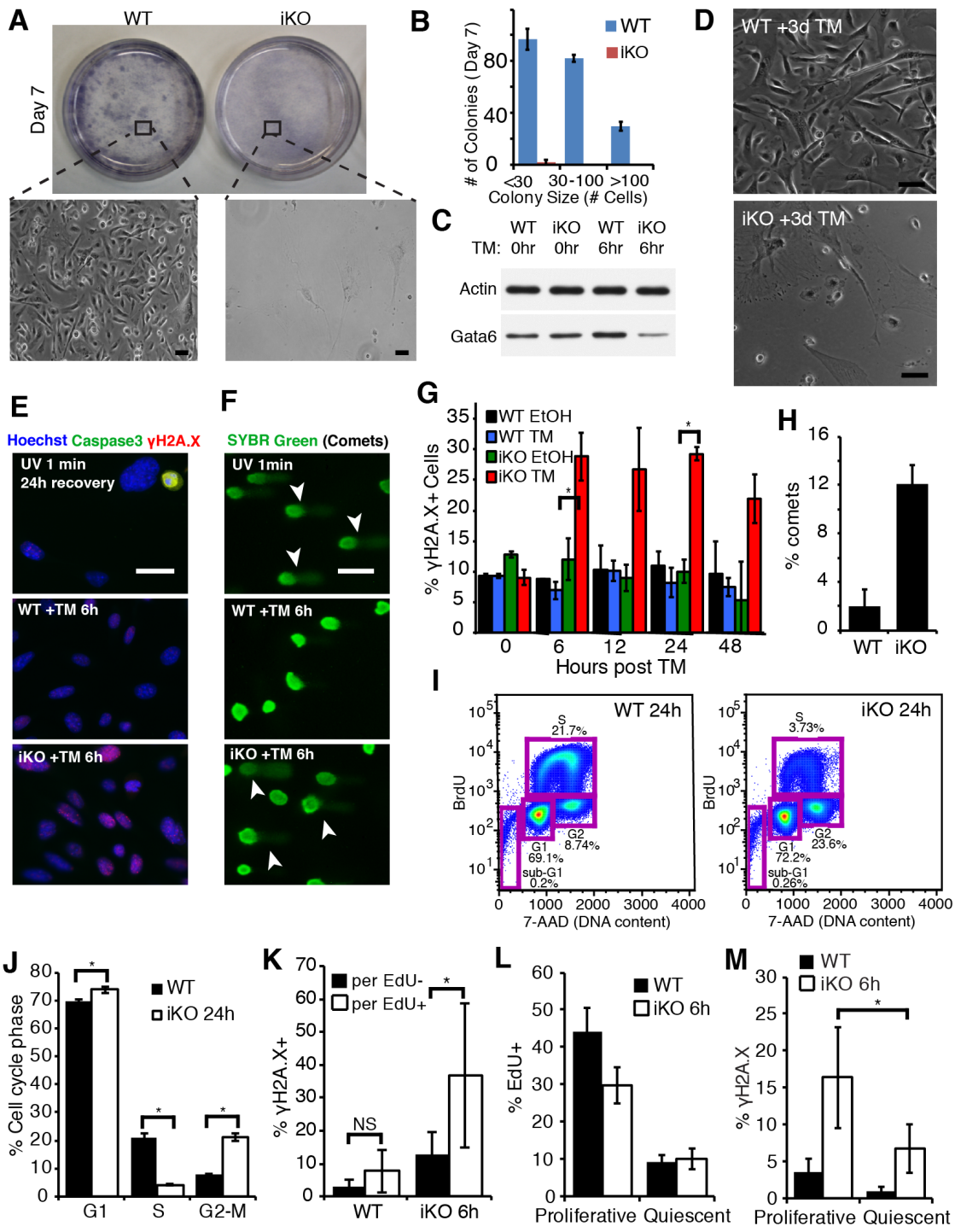
In conclusion loss of *Gata6* at anagen induced rapid DNA damage, apoptosis and decreased proliferation in the hair follicle progenitors throughout the matrix, providing an explanation for early cessation of production in terminal differentiated lineages and collapse of hair follicle into premature catagen.

### *3.7 Cultured keratinocytes acquire proliferation associated DNA-damage following Gata6 loss*

To better understand the intrinsic cellular responses that occur upon *Gata6* loss, we examined cultured keratinocytes isolated from the skin epithelium of *Gata6* WT and iKO newborn mice. Induction of iKO by TM administration to pups prior to cell plating resulted in complete loss of colony formation and growth abilities of iKO relative to WT cells (Fig 3.9A,B). While large colonies are seeded by keratinocyte

**Figure 3.9** Proliferation-induced DNA damage in keratinocytes results in cell cycle arrest and cell death. (**A**) Colony formation of WT and TM induced *Gata6* iKO keratinocytes stained with hematoxylin. Primary keratinocytes were isolated from induced iKO newborn mice, plated on feeder cells, and stained with hematoxylin at day 7 after plating (top). Phase contrast images of cells are shown below. Scale bars: 30 μm. (**B**) Quantification of colony formation from (**A**), average ± SD from n=3 mice in each genotype. (**C**) Western blot of WT and iKO cultured keratinocytes after 0 or 6 hours of tamoxifen induction, blotted with actin and GATA6 antibodies, show a decrease in GATA6 protein levels after 6 hours. (**D**) Phase contrast images of WT and *Gata6* iKO keratinocytes 3 days after induction with TM (n > 3 per genotype). (**E-H**) Images and quantification of DNA damage in cultured keratinocytes based on staining for DNA damage marker γH2A.X (**E and G**) and comet assay (**F and H**). Arrowheads in (**F**) indicate DNA damaged cell nuclei with comet shape. Quantification of γH2A.X (**G**) represents average ± SD from at least 300 cells per condition from 3 experiments using different cell lines . *Unpaired t-test P-values: 6h=0.01 and 24h=0.004. **(H)** Quantification of comet assay represents average ± SD (n=3). (**I-J**) FACS cell cycle profiles and quantification show that Gata6 iKO keratinocytes show decreased proliferation and G2-M arrest. (**J**) Quantification of FACS cell cycle analysis represent average ± SD (n=3 per genotype). *Unpaired t-test P-values: G1 = 0.03, S = $5x10^{-4}$, G2-M = $7x10^{-4}$. (**K**) Quantification of the frequency of γH2A.X+ staining in keratinocytes within S phase (EdU+) or another cell cycle stage (EdU-) following a 20 minute EdU pulse labeling (average ± SD, n=5). *Unpaired t-test P-values WT=0.1,

iKO= 0.05. Mann-Whitney U test P-values: WT=0.09, iKO=0.03. (**L-M**) Cultured keratinocytes were made quiescent by gradual serum depletion. (**L**) Quantification of EdU+ cells in proliferative and serum depleted quiescent cells (average ± SD, n=4). (**M**) Quantification of γH2A.X+ keratinocytes during proliferative and quiescent conditions (average ± SD, n=4). *Unpaired t-test P-value = 0.04. Mann-Whitney U test P-value=0.06.


A
WT
iKO
Day 7
B
# of Colonies (Day 7)
WT
iKO
<30
30-100
>100
Colony Size (# Cells)
C
WT iKO WT iKO
TM: 0hr 0hr 6hr 6hr
Actin
Gata6
D
WT +3d TM
iKO +3d TM
E
Hoechst Caspase3 γH2A.X
UV 1 min
24h recovery
WT +TM 6h
iKO +TM 6h
F
SYBR Green (Comets)
UV 1min
WT +TM 6h
iKO +TM 6h
G
WT EtOH
WT TM
iKO EtOH
iKO TM
% γH2A.X+ Cells
Hours post TM
H
% comets
WT
iKO
I
WT 24h
iKO 24h
BrdU
7-AAD (DNA content)
S
21.7%
G1
69.1%
G2
8.74%
sub-G1
0.2%
S
3.73%
G1
72.2%
G2
23.6%
sub-G1
0.26%
J
% Cell cycle phase
WT
iKO 24h
G1
S
G2-M
K
per EdU-
per EdU+
% γH2A.X+
NS
WT
iKO 6h
L
WT
iKO 6h
% EdU+
Proliferative
Quiescent
M
WT
iKO 6h
% γH2A.X
Proliferative
Quiescent

stem cells, the subsequent cultured cells are mixtures of rare stem cells and frequent progenitor cells (Barrandon & Green, 1987). The loss of colony formation ability could be explained by an effect of *Gata6* on stem cells themselves or on their early progenitor cells. Thus, to test more directly the possible role in progenitor cells, we established cell lines from WT and iKO mice prior to TM induction and induced the *Gata6* loss in the cultured cells. Within 3 days post induction, cells were largely lost from the dish (Fig 3.9D), suggesting a rapid depletion of the cultured progenitor cells upon *Gata6* loss. To examine the early cellular responses to *Gata6* loss, we asked how quickly is GATA6 protein depleted post-induction by performing Western blots. We found that GATA6 is substantially reduced as early as 6 hours following TM addition to the culture medium (Fig 3.9C). As with the hair follicle matrix cells *in vivo*, iKO cultured keratinocytes rapidly acquired DNA damage indicated by γH2A.X foci staining, as early as 6 hours post induction (Fig 3.9E,G). We also confirmed the presence of DNA damage in keratinocytes by comet assays, using WT cells with 1-minute high UV dose irradiation as positive controls (Fig 3.9F,H). Apoptosis was not detectable in iKO cells 6 hours post-induction, although it was readily detectable in our control WT cells treated with high UV doses and cultured for 24 hours (Fig 3.9E). These results indicated that DNA damage is an early and rapid response to *Gata6* loss. Cell cycle analysis of keratinocytes at 24 hours post TM induction displayed decreased proliferation in response to *Gata6* loss. This was indicated by a dramatic reduction in the fraction of cells in S-phase (G1-phase was also somewhat decreased) of the cycle in the *Gata6* iKO, and an increase in the G2/M-phase. These data suggest cell cycle arrest occurs in response of the observed DNA damage induced by *Gata6*

loss (Fig 3.9I,J). These effects on the cell cycle were also detectable in cell culture using a knockdown approach with *Gata6*-specific shRNA (Fig 3.10).

To examine the possible relationship between replication and DNA damage in response to *Gata6* iKO, we co-stained EdU labeled cells with γH2A.X and found a significantly greater proportion of γH2A.X+ cells associated with EdU+ cells than EdU- cells (Fig 3.9K). Furthermore, to test if proliferation is necessary for *Gata6* iKO cells to acquire DNA damage, we attempted to make cultured keratinocytes quiescent. A decrease in EdU labeling confirmed increased quiescence of WT and iKO cells upon gradual serum withdrawal (Fig 3.9L). We found DNA damage indicated by γH2A.X staining was decreased in quiescent cells compared to proliferative cells (Fig 3.9M). The residual DNA damage observed in the serum-deprived iKO cells may be attributable to the incomplete cell cycle arrest, as indicated by the remaining ~10% EdU labeled cells. These data suggested that *Gata6* plays a role in protecting proliferative keratinocytes from DNA damage, cell cycle arrest, and eventual cell death with loss from the culture dish. These results were in line with the effects of *Gata6* loss on the hair follicle matrix cells in vivo, suggesting that cultured keratinocytes are a good model to study in more details mechanisms of *Gata6* action in skin epithelial cells.

### *3.8 Genome maintenance factors are downstream targets of Gata6*

To understand the downstream mechanisms by which *Gata6* performs its cellular function in protecting the genome from DNA damage and cell cycle arrest in proliferative skin epithelial cells we performed mRNA sequencing (RNA-seq). For

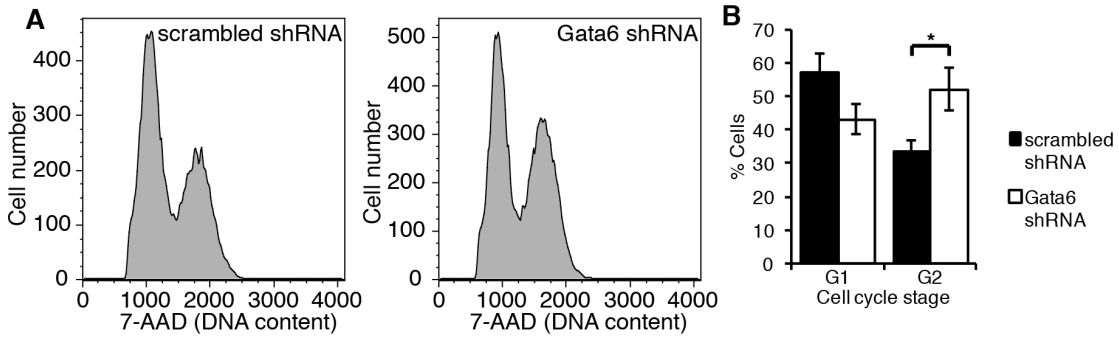


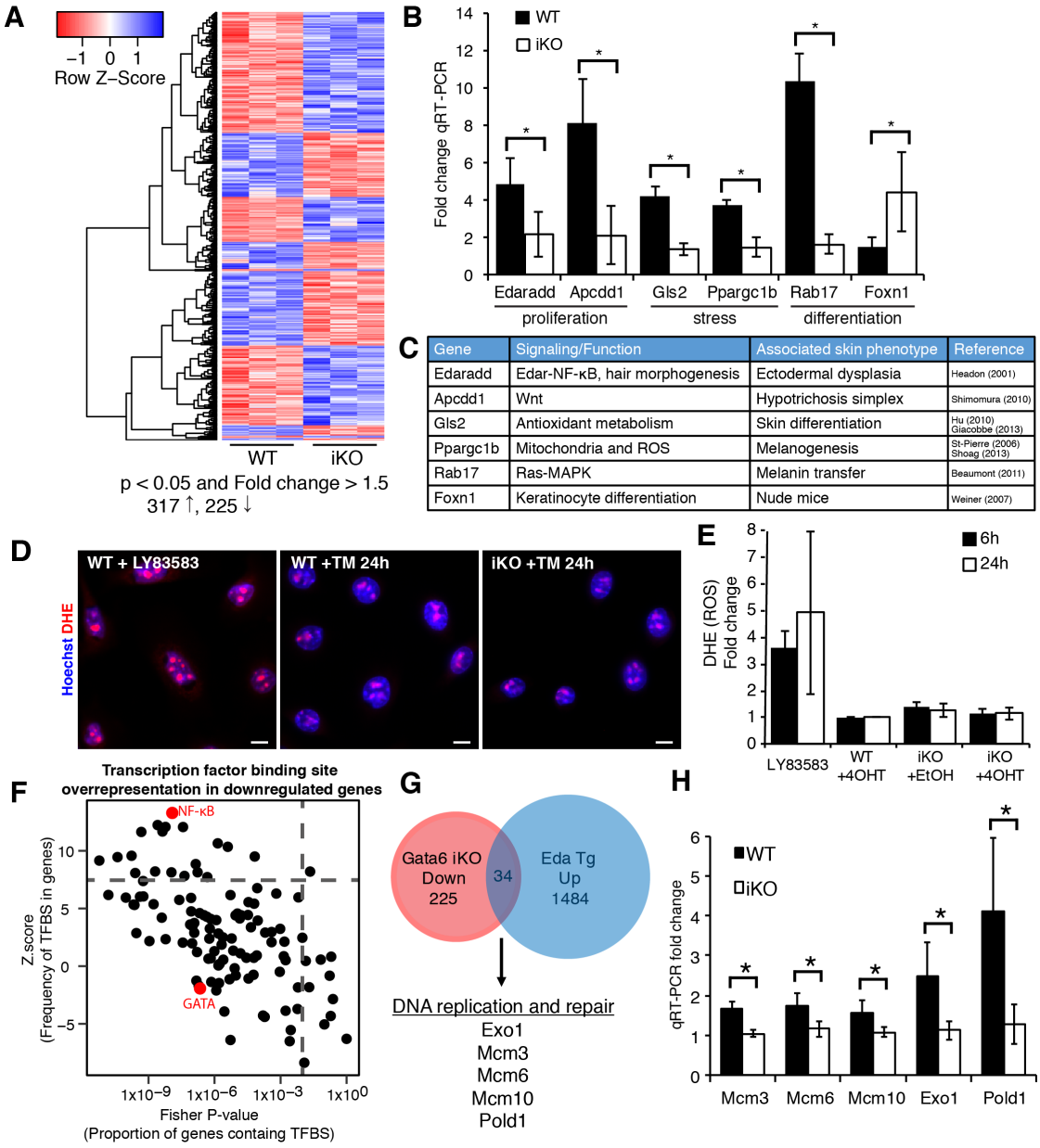

**Figure 3.10** *Gata6* shRNA knockdown in keratinocytes causes cell cycle arrest. (A) FACS cell cycle profiles of cells transfected with scrambled shRNA or *Gata6* shRNA. (B) Quantification of cell cycle stages of keratinocytes treated with scrambled or *Gata6* shRNA (n=5).

this analysis, we used RNA purified from independent iKO and WT keratinocyte lines (3 lines each) 6 hours after TM induction. 542 genes were differentially expressed by > 1.5-fold (adjusted *P*-value < 0.05) in *Gata6* iKO compared to WT keratinocytes, with 317 up-regulated genes and 225 down-regulated genes (Figs 3.11A and 3.12). We selected a number of candidate genes whose mutants have similar degenerative hair follicle phenotypes to those observed in *Gata6* iKO mice and confirmed their expression changes by qRT-PCR (Fig 3.11B,C). Given the observed DNA damage phenotype observed both *in vivo* and *in vitro*, we were initially drawn to the significantly down-regulated genes involved in antioxidant function such as *Gls2*, *Gclc*, and *Gsr*, and *Gstp1*, which are all enzymes involved in the synthesis of the key antioxidant glutathione. However, we examined reactive oxygen species (ROS) levels with dihydroethidium staining of cells and found no significant change in *Gata6* iKO keratinocytes compared to WT (Fig 3.11D,E). Given the association of DNA-damage induced by *Gata6* loss with S-phase, we suspected a possible implication of replication-induced stress (Berti & Vindigni, 2016).

To better understand the various downstream processes and pathways *Gata6* may be affecting, we conducted gene ontology and transcription factor binding site enrichment analyses on the differentially expressed genes. Gene ontology analyses revealed that among the down-regulated genes, there was an overrepresentation of genes related to cell cycle and cell proliferation, as well as DNA metabolism, which included a number of genes related to DNA replication as well as repair (Table 3.1). Additionally, we conducted transcription factor binding site enrichment analyses on

**Figure 3.11** Genome-wide transcriptional changes following *Gata6* depletion. (**A**) Heatmap of differentially expressed genes (adjusted P-value < 0.05 and fold change > 1.5) from RNA-seq (n=3 different WT and iKO cell lines). (**B**) qRT-PCR validation of candidate genes selected from RNA-seq analysis using RNA from cultured cells (average ± SD, n=4). *Unpaired t-test P-values: *Edaradd*=0.03, *Apcdd1*=0.005, *Gls2*=$7x10^{-5}$, *Ppargc1b*=$3x10^{-4}$, *Rab17*=$2x10^{-5}$, *Foxn1*=0.03. Mann-Whitney U test P-values: *Edaradd*=0.06, *Apcdd1*=0.03, *Gls2*=0.03, *Ppargc1b*=0.03, *Rab17*=0.03, *Foxn1*=0.03. (**C**) Table summarizing selection of candidate genes from (**B**). Genes were selected based on known function or skin phenotype related to Gata6 iKO phenotype. (**D**) Staining of ROS by DHE. LY83583 treated cells served as positive control with elevated ROS. (**E**) Quantification of fold change in ROS at 6 and 24 hours after TM compared to WT, based on fluorescence intensity of DHE (average ± SD, n=2). (**F**) Analysis of transcription factor binding site overrepresentation (oPOSSUM) in differentially expressed genes from RNA-seq. Points represent transcription factors plotted as Z.score of the frequency of transcription factor binding site occurrences in the promoters of downregulated genes compared to the background rate of occurrences in all gene promoters and Fisher P-value of the proportion of downregulated gene promoters containing each transcription factor binding site compared to the frequency in all gene promoters. (**G**) Comparison of *Gata6* iKO downregulated genes with genes upregulated in *Eda* transgenic (Tg) skin. Genes related to DNA replication and repair are found in the set of genes shared between the two datasets. (**H**) qRT-PCR of selected genes found to be overlapping between *Gata6* iKO and *Eda* transgenic analyzed in *Gata6* WT and iKO keratinocytes (average ± SD,

n=4). *Unpaired t-test P-values: *Mcm3*=0.001, *Mcm6*=0.02, *Mcm10*=0.04, *Exo1*=0.02, *Pold1*=0.02. Mann-Whitney U test P-values: *Mcm3*=0.03, *Mcm6*=0.03, *Mcm10*=0.03, *Exo1*=0.03, *Pold1*=0.06.




| Gene | Signaling/Function | Associated skin phenotype | Reference |
|---|---|---|---|
| Edaradd | Edar-NF-κB, hair morphogenesis | Ectodermal dysplasia | Headon (2001) |
| Apcdd1 | Wnt | Hypotrichosis simplex | Shimomura (2010) |
| Gls2 | Antioxidant metabolism | Skin differentiation | Hu (2010) Giacobbe (2013) |
| Ppargc1b | Mitochondria and ROS | Melanogenesis | St-Pierre (2006) Shoag (2013) |
| Rab17 | Ras-MAPK | Melanin transfer | Beaumont (2011) |
| Foxn1 | Keratinocyte differentiation | Nude mice | Weiner (2007) |

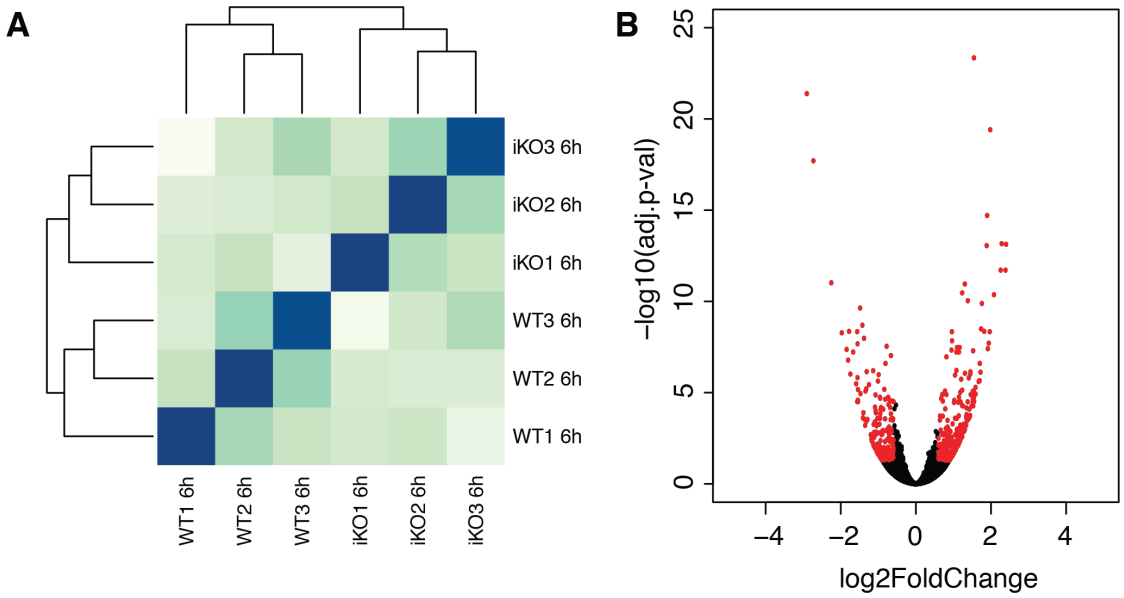



**Figure 3.12** Differential expression of *Gata6* iKO RNA-seq.(A) Heatmap showing Euclidean distances between samples from RNA-seq (n=3 per genotype). (B) Volcano plot of genes from RNA-seq analysis with differentially expressed genes (adjusted P-value <0.05 and fold change > 1.5) highlighted in red.

**Table 3.1** Gene ontology analysis of *Gata6* iKO differentially expressed genes. (A) Overrepresented gene ontology terms from *Gata6* iKO downregulated genes. (B) Overrepresented gene ontology terms from *Gata6* iKO upregulated genes.

**A**

| GO: Gata6 iKO downregulated genes | | |
|---|---|---|
| GO:Biological Process Term | P-value | Genes (Fold Change) |
| Developmental processes | $7.4 \times 10^{-4}$ | ADAMTS1 (-2.1x), CEACAM12 (-1.9x), CHEK1 (-1.7x), COL12A1 (-1.9x), DLK2 (-2.1x), DLX2 (-2x), FARP2 (-1.5x), FIGF (-2x), FOXO1 (-1.7x), HMX1 (-2.5x), HS3ST3B1 (-1.9x), IL6ST (-1.6x), IRX1 (-1.7x), LDLRAD3 (-1.6x), LFNG (-1.9x), LRRC33 (-1.9x), MET (-1.6x), MID1 (-2.5x), NEURL1B (-1.9x), NINJ1 (-1.8x), NOG (-2x), NTM (-2.8x), NTN4 (-1.8x), OTUD1 (-2x), PAX8 (-1.9x), PCDH7 (-1.6x), PRL2C5 (-6.6x), PRL8A1 (-1.9x), PSG28 (-1.9x), RBL1 (-1.5x), RBMX (-1.7x), RPS6KA6 (-2.1x), RTN4R (-1.7x), SIX5 (-1.7x), TBX1 (-3.6x), TCP11L1 (-1.7x), TIGD2 (-1.5x) |
| Cell cycle | $6.9 \times 10^{-3}$ | 1700017B05RIK (-1.9x), CHEK1 (-1.7x), E2F1 (-1.8x), EXO1 (-1.7x), FIGF (-2x), FOXO1 (-1.7x), ING5 (-1.5x), MCM3 (-1.6x), MCM6 (-1.7x), MET (-1.6x), NDN (-1.9x), OTUD1 (-2x), POLD1 (-1.6x), RBL1 (-1.5x), RPS6KA6 (-2.1x), TNS1 (-2.3x), TRP63 (-1.6x), UHRF1 (-1.7x) |
| DNA metabolism | $2.5 \times 10^{-2}$ | DNMT3B (-1.6x), DTYMK (-1.5x), EXO1 (-1.7x), MCM3 (-1.6x), MCM6 (-1.7x), POLD1 (-1.6x), TRP63 (-1.6x), UNG (-1.6x) |
| Tumor suppressor | $5.1 \times 10^{-2}$ | ING5 (-1.5x), RBL1 (-1.5x), TNS1 (-2.3x), TRP63 (-1.6x) |
| Cell adhesion | $6.4 \times 10^{-2}$ | CEACAM12 (-1.9x), COL12A1 (-1.9x), COL16A1 (-2.4x), CTNNAL1 (-1.9x), LGALS7 (-3x), LRRC32 (-1.9x), NINJ1 (-1.8x), NTM (-2.8x), PCDH7 (-1.6x), PSG28 (-1.9x), TNS1 (-2.3x) |
| Induction of apoptosis | $6.4 \times 10^{-2}$ | CIDEA (-2.5x), LGALS7 (-3x), TNF (-1.9x), TNS1 (-2.3x), TRP63 (-1.6x) |
| Amino acid metabolism | $7.0 \times 10^{-2}$ | CCBL2 (-1.6x), CTH (-1.8x), GLS2 (-2.1x), HPDL (-1.6x), SHMT1 (-1.5x), SLC7A5 (-1.7x) |

**B**

| GO: Gata6 iKO upregulated genes | | |
|---|---|---|
| GO:Biological Process Term | P-value | Genes (Fold Change |
| Cell communication | $2.4 \times 10^{-4}$ | 3110039M20RIK (2.0x), ADAM22 (3.3x), ADRB1 (2.1x), ARTN (3.5x), BTC (2.0x), CDH2 (3.3x), CHRNA5 (1.9x), CLCF1 (1.8x), COL6A2 (2.3x), CRABP2 (2.9x), CYR61 (1.7x), EFEMP2 (4.8x), ESR1 (15.3x), FBLN5 (2.1x), FOXN1 (1.9x), HBEGF (1.6x), IL24 (1.8x), MSLNL (2.0x), NOTCH3 (2.0x), PCDHGA10 (2.0x), PCDHGA11 (5.2x), PCDHGA12 (4.2x), PCDHGB8 (2.4x), PDGFC (2.2x), PSTPIP1 (2.0x), PTPRU (1.9x), PVRL2 (1.6x), PVRL4 (1.6x), SEMA4G (2.2x), SLC1A1 (2.2x), STAB1 (1.8x), TSPAN14 (1.6x), WNT11 (2.1x), WNT7A (1.9x) |
| Cell proliferation and differentiation | $2.7 \times 10^{-4}$ | 3110039M20RIK (2.0x), ABTB2 (1.6x), AKT3 (1.9x), BTC (2.0x), CDKN1A (1.6x), CGREF1 (2.3x), CYR61 (1.7x), ESR1 (15.3x), FOXN1 (1.9x), FRK (1.7x), GADD45B (1.8x), HBEGF (1.6x), IFI203 (2.2x), IFI204 (1.7x), INHBB (2.9x), JMJD7 (2.7x), KLK8 (2.5x), MST1R (1.6x), NDRG2 (2.6x), NOTCH3 (2.0x), PDGFC (2.2x), PLA2G4B (1.8x), PYHIN1 (2.0x), S1PR3 (2.3x), SDC3 (1.7x), TNS3 (1.6x), ZFP182 (1.8x), ZFP37 (2.0x), ZFP651 (1.5x) |
| Cell structure and motility | $4.5 \times 10^{-3}$ | 3110039M20RIK (2.0x), AMOTL2 (1.5x), ANK1 (2.3x), ANK3 (1.7x), ARHGAP8 (3.0x), ARTN (3.5x), ASAP3 (1.8x), BC021767 (1.6x), CGN (1.9x), CLDN23 (2.4x), CLDN4 (1.7x), COL6A2 (2.3x), CYR61 (1.7x), DBN1 (1.9x), EPB4.9 (3.8x), ESR1 (15.3x), FOXN1 (1.9x), GM14137 (1.6x), KIF5C (3.4x), LYSMD4 (1.7x), MST1R (1.6x), NOTCH3 (2.0x), PHACTR2 (2.1x), PLEKHG6 (1.8x), PRICKLE1 (2.2x), REM2 (2.2x), RHOB (1.7x), SDC3 (1.7x) |
| Developmental processes | $6.7 \times 10^{-3}$ | 3110039M20RIK (2.0x), AKT3 (1.9x), AMOTL2 (1.5x), ARTN (3.5x), CRABP2 (2.9x), CYR61 (1.7x), D7ERTD443E (2.1x), DMRT2 (5.3x), EFEMP2 (4.8x), EMX2 (2.2x), EOMES (2.1x), ESR1 (15.3x), FBLN5 (2.1x), FIGNL2 (1.6x), FOXN1 (1.9x), FRK (1.7x), GATA6 (2.3x), HOXB6 (1.8x), HOXB7 (1.5x), HOXB8 (2.7x), HOXB9 (1.7x), HS3ST1 (1.9x), INHBB (2.9x), KDM5D (46.4x), KLK8 (2.5x), LRP1 (1.7x), MDFI (1.7x), MSL3L2 (2.0x), MSLNL (2.0x), MST1R (1.6x), NFAT5 (1.6x), NFE2L3 (2.1x), NKX3-1 (2.7x), NOTCH3 (2.0x), PDGFC (2.2x), PODNL1 (2.4x), PRICKLE1 (2.2x), PRSS8 (1.9x), PSTPIP1 (2.0x), RELN (2.9x), SMPDL3B (1.8x), SPRED3 (2.0x), TGM1 (2.7x), WNT11 (2.1x), WNT7A (1.9x), ZFP37 (2.0x) |
| Cell adhesion | $1.2 \times 10^{-2}$ | ASAP3 (1.8x), CD209C (2.0x), CD97 (1.9x), CDH2 (3.3x), CEACAM19 (1.7x), COL6A2 (2.3x), MSLNL (2.0x), PCDHGA10 (2.0x), PCDHGA11 (5.2x), PCDHGA12 (4.2x), PCDHGB8 (2.4x), PLEKHG6 (1.8x), PTPRU (1.9x), RAP1GAP2 (1.9x), SDC3 (1.7x), SIPA1L2 (1.6x), TNS3 (1.6x), TSPAN14 (1.6x) |
| Cell cycle control | $1.4 \times 10^{-2}$ | 3110039M20RIK (2.0x), AKT3 (1.9x), CDK5R1 (1.7x), CDKN1A (1.6x), CYR61 (1.7x), ESR1 (15.3x), FOXN1 (1.9x), FRK (1.7x), GADD45B (1.8x), GAS2 (1.9x), KLK8 (2.5x), MST1R (1.6x), TNS3 (1.6x) |
| mRNA transcription | $4.1 \times 10^{-2}$ | 3110039M20RIK (2.0x), 5730507C01RIK (1.7x), BCL3 (1.9x), DENND3 (2.9x), EMX2 (2.2x), EOMES (2.1x), ESR1 (15.3x), FOXN1 (1.9x), GATA6 (2.3x), GLIS1 (2.0x), GM14403 (1.8x), GRHL3 (1.8x), HDAC6 (1.7x), HOXB6 (1.8x), HOXB7 (1.5x), HOXB8 (2.7x), HOXB9 (1.7x), IFI203 (2.2x), IFI204 (1.7x), IRF5 (2.2x), IRF7 (1.9x), KDM5D (46.4x), MDFI (1.7x), MSL3L2 (2.0x), NFAT5 (1.6x), NKX3-1 (2.7x), NOL3 (2.1x), OCEL1 (1.6x), PLEKHF1 (3.2x), PYHIN1 (2.0x), SNAI3 (2.3x), SOX2 (2.3x), SP6 (1.6x), ZFP182 (1.8x), ZFP37 (2.0x), ZFP651 (1.5x), ZFP704 (2.3x) |
| Cell adhesion-mediated signaling | $4.9 \times 10^{-2}$ | ADAM22 (3.3x), CDH2 (3.3x), NOTCH3 (2.0x), PCDHGA10 (2.0x), PCDHGA11 (5.2x), PCDHGA12 (4.2x), PCDHGB8 (2.4x), PSTPIP1 (2.0x), PTPRU (1.9x), PVRL2 (1.6x), PVRL4 (1.6x), TSPAN14 (1.6x) |
| Phospholipid metabolism | $5.8 \times 10^{-2}$ | ABCA1 (2.5x), JMJD7 (2.7x), PLA2G15 (1.9x), PLA2G4B (1.8x), PLD1 (1.6x), SYNJ2 (2.2x), TNS3 (1.6x) |

the promoters of the genes changed in response to *Gata6* loss using the oPOSSUM database (Ho Sui *et al*, 2005). Interestingly, the most significantly overrepresented transcription factor conserved binding site in the putative promoters of *Gata6* down-regulated genes was that of NF-κB (Fig 3.11F and Table 3.2). NF-κB has an established role in regulating proliferation, DNA repair, and apoptosis especially in response to stress (Janssens & Tschopp, 2006). Furthermore, inhibition of the NF-κB pathway has been shown to have a phenotype in hair follicles resembling the premature degeneration seen in the of *Gata6* iKO (Sayama *et al*, 2010). Together, this suggested the possibility that *Gata6* may be maintaining hair follicle progenitor cells indirectly, through activation of the NF-κB pathway, thus preventing accumulation of DNA damage and apoptosis.

One of the genes we found to be down-regulated in our RNA-seq analysis was *Edaradd* (Fig 3.11B, C), which is linked with *Eda* and NF-κB signaling and has established roles in hair morphogenesis and in the hair cycle (Cui & Schlessinger, 2006; Mustonen *et al*, 2003; Kloepper *et al*, 2014). We also found the NF-κB activator *Tnfα* was downregulated as well in response to *Gata6* loss. *Tnfα* has also been reported to have effects on hair follicle growth (Tong & Coulombe, 2006; Chen *et al*, 2015). To address which genes downstream of *Gata6* may be regulated by *Edar*-NF-κB signaling, we compared the genes down-regulated by *Gata6* loss with those that were previously found to be up-regulated by *Eda* overexpression (Cui *et al*, 2006). Interestingly, among the 34 genes we found to be shared between the two sets (Table 3.3), a handful were involved in DNA replication and repair and showed expected downregulation of gene expression by qRT-PCR (Fig 3.11H). Loss of these genes

**Table 3.2** oPOSSUM analysis of overrepresented transcription factor binding sites in *Gata6* iKO differentially expressed genes. (A) Top ten overrepresented transcription factor binding sites identified in *Gata6* iKO downregulated genes. (B) Top ten overrepresented transcription factor binding sites identified in *Gata6* iKO upregulated genes.

**A**

| Overrepresented TFBS in Gata6 iKO downregulated genes | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TF | JASPAR ID | Target gene hits | Target gene non-hits | Background gene hits | Background gene non-hits | Target TFBS hits | Target TFBS nucleotide rate | Background TFBS hits | Background TFBS nucleotide rate | Z-score | Fisher (P-value) |
| NFKB1 | MA0105.1 | 107 | 107 | 9198 | 20149 | 273 | 0.00719 | 19859 | 0.00564 | 13.282 | $1.2x10^{-6}$ |
| Mycn | MA0104.2 | 161 | 53 | 16332 | 13015 | 849 | 0.0203 | 68924 | 0.0178 | 12.232 | $2.6x10^{-9}$ |
| Klf4 | MA0039.2 | 185 | 29 | 20377 | 8970 | 2360 | 0.0565 | 202434 | 0.0523 | 12.069 | $5.9x10^{-9}$ |
| EBF1 | MA0154.1 | 180 | 34 | 19841 | 9506 | 1471 | 0.0352 | 123513 | 0.0319 | 12.06 | $3.7x10^{-8}$ |
| Arnt::Ahr | MA0006.1 | 194 | 20 | 21968 | 7379 | 2990 | 0.0429 | 254193 | 0.0394 | 11.673 | $4.3x10^{-9}$ |
| Myc | MA0147.1 | 164 | 50 | 16509 | 12838 | 848 | 0.0203 | 69281 | 0.0179 | 11.635 | $4.7x10^{-10}$ |
| Zfp423 | MA0116.1 | 142 | 72 | 14730 | 14617 | 603 | 0.0216 | 49975 | 0.0194 | 10.648 | $1.4x10^{-6}$ |
| Esrrb | MA0141.1 | 173 | 41 | 19606 | 9741 | 1178 | 0.0338 | 100122 | 0.031 | 10.346 | $4.0x10^{-6}$ |
| Egr1 | MA0162.1 | 141 | 73 | 12980 | 16367 | 539 | 0.0142 | 44110 | 0.0125 | 9.57 | $1.7x10^{-10}$ |
| RELA | MA0107.1 | 130 | 84 | 13922 | 15425 | 481 | 0.0115 | 38861 | 0.01 | 9.504 | $6.7x10^{-5}$ |

**B**

| Overrepresented TFBS in Gata6 iKO upregulated genes | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TF | JASPAR ID | Target gene hits | Target gene non-hits | Background gene hits | Background gene non-hits | Target TFBS hits | Target TFBS nucleotide rate | Background TFBS hits | Background TFBS nucleotide rate | Z-score | Fisher (P-value) |
| SP1 | MA0079.2 | 261 | 37 | 19074 | 10273 | 3119 | 0.0548 | 168476 | 0.0435 | 41.656 | $9.3x10^{-19}$ |
| Klf4 | MA0039.2 | 263 | 35 | 20377 | 8970 | 3628 | 0.0637 | 202434 | 0.0523 | 38.749 | $1.8x10^{-14}$ |
| MZF1_1-4 | MA0056.1 | 285 | 13 | 24306 | 5041 | 9551 | 0.101 | 561826 | 0.0871 | 36.34 | $1.5x10^{-11}$ |
| MZF1_5-13 | MA0057.1 | 271 | 27 | 21759 | 7588 | 3852 | 0.0677 | 222525 | 0.0575 | 32.991 | $2.6x10^{-13}$ |
| Tcfcp2l1 | MA0145.1 | 261 | 37 | 20290 | 9057 | 2461 | 0.0605 | 142543 | 0.0516 | 30.6 | $8.7x10^{-14}$ |
| Zfx | MA0146.1 | 242 | 56 | 16844 | 12503 | 1696 | 0.0417 | 96083 | 0.0348 | 28.664 | $3.3x10^{-18}$ |
| INSM1 | MA0155.1 | 229 | 69 | 16347 | 13000 | 1313 | 0.0277 | 73032 | 0.0226 | 25.545 | $3.2x10^{-14}$ |
| ZNF354C | MA0130.1 | 285 | 13 | 24684 | 4663 | 9348 | 0.0986 | 575386 | 0.0892 | 24.722 | $4.3x10^{-10}$ |
| EBF1 | MA0154.1 | 250 | 48 | 19841 | 9506 | 2088 | 0.0367 | 123513 | 0.0319 | 20.475 | $1.7x10^{-10}$ |
| PPARG | MA0065.2 | 225 | 73 | 16901 | 12446 | 1138 | 0.03 | 66652 | 0.0258 | 19.772 | $1.0x10^{-10}$ |

**Table 3.3** Table of the 34 genes identified as overlapping between the *Gata6* iKO downregulated and *Eda* transgenic upregulated datasets.

| ID | baseMean | log2FoldCh | lfcSE | stat | pvalue | padj |
|---|---|---|---|---|---|---|
| 2810417H13Rik | 5080.00 | -0.75 | 0.17 | -4.35 | 1.35E-05 | 9.20E-04 |
| Bhlhb9 | 23.66 | -1.00 | 0.28 | -3.58 | 3.39E-04 | 1.12E-02 |
| Cdca7 | 3317.87 | -0.81 | 0.13 | -6.10 | 1.03E-09 | 2.50E-07 |
| Chn2 | 95.14 | -1.97 | 0.29 | -6.76 | 1.39E-11 | 5.25E-09 |
| Col12a1 | 1662.06 | -0.90 | 0.21 | -4.25 | 2.13E-05 | 1.33E-03 |
| Col4a1 | 8873.69 | -0.78 | 0.25 | -3.11 | 1.90E-03 | 3.67E-02 |
| Creb5 | 298.91 | -0.61 | 0.19 | -3.19 | 1.44E-03 | 3.11E-02 |
| Cth | 1865.13 | -0.84 | 0.25 | -3.40 | 6.70E-04 | 1.87E-02 |
| Cxcl14 | 878.11 | -0.62 | 0.20 | -3.11 | 1.86E-03 | 3.63E-02 |
| Dlx2 | 1162.37 | -1.03 | 0.27 | -3.78 | 1.56E-04 | 6.54E-03 |
| Dtymk | 2500.94 | -0.62 | 0.17 | -3.73 | 1.90E-04 | 7.47E-03 |
| Edaradd | 513.19 | -0.95 | 0.18 | -5.28 | 1.27E-07 | 1.78E-05 |
| Exo1 | 1859.63 | -0.77 | 0.18 | -4.29 | 1.78E-05 | 1.14E-03 |
| Farp2 | 444.43 | -0.63 | 0.19 | -3.28 | 1.02E-03 | 2.55E-02 |
| Foxo1 | 1831.44 | -0.73 | 0.21 | -3.52 | 4.25E-04 | 1.36E-02 |
| Glrb | 23.93 | -1.54 | 0.30 | -5.15 | 2.67E-07 | 3.09E-05 |
| Gyltl1b | 270.74 | -2.90 | 0.28 | -10.37 | 3.28E-25 | 4.10E-22 |
| Iars2 | 5070.40 | -0.68 | 0.14 | -4.98 | 6.23E-07 | 6.48E-05 |
| Ica1 | 26.69 | -1.03 | 0.29 | -3.51 | 4.45E-04 | 1.40E-02 |
| Mcm10 | 2234.25 | -0.64 | 0.18 | -3.45 | 5.68E-04 | 1.68E-02 |
| Mcm3 | 8781.65 | -0.67 | 0.14 | -4.63 | 3.66E-06 | 2.97E-04 |
| Mcm6 | 9302.96 | -0.78 | 0.12 | -6.49 | 8.73E-11 | 2.87E-08 |
| Oip5 | 599.89 | -0.71 | 0.16 | -4.40 | 1.08E-05 | 7.65E-04 |
| Osbpl10 | 95.35 | -0.89 | 0.27 | -3.31 | 9.32E-04 | 2.39E-02 |
| Pold1 | 1814.51 | -0.67 | 0.21 | -3.19 | 1.42E-03 | 3.09E-02 |
| Sirpa | 1142.72 | -1.01 | 0.18 | -5.69 | 1.25E-08 | 2.32E-06 |
| Sox21 | 407.53 | -1.38 | 0.21 | -6.65 | 2.85E-11 | 1.05E-08 |
| Tgif2 | 661.30 | -0.68 | 0.22 | -3.14 | 1.71E-03 | 3.48E-02 |
| Tnf | 74.27 | -0.89 | 0.28 | -3.21 | 1.33E-03 | 3.02E-02 |
| Trip13 | 2142.01 | -0.62 | 0.16 | -3.83 | 1.26E-04 | 5.48E-03 |
| Trp63 | 11360.33 | -0.66 | 0.11 | -6.27 | 3.56E-10 | 9.27E-08 |
| Uhrf1 | 8226.73 | -0.76 | 0.19 | -3.96 | 7.56E-05 | 3.72E-03 |
| Ung | 746.84 | -0.66 | 0.21 | -3.08 | 2.10E-03 | 3.94E-02 |
| Vsig8 | 191.07 | -0.91 | 0.30 | -3.04 | 2.40E-03 | 4.29E-02 |

could potentially explain the observed DNA damage induced by *Gata6* iKO in proliferative cells.

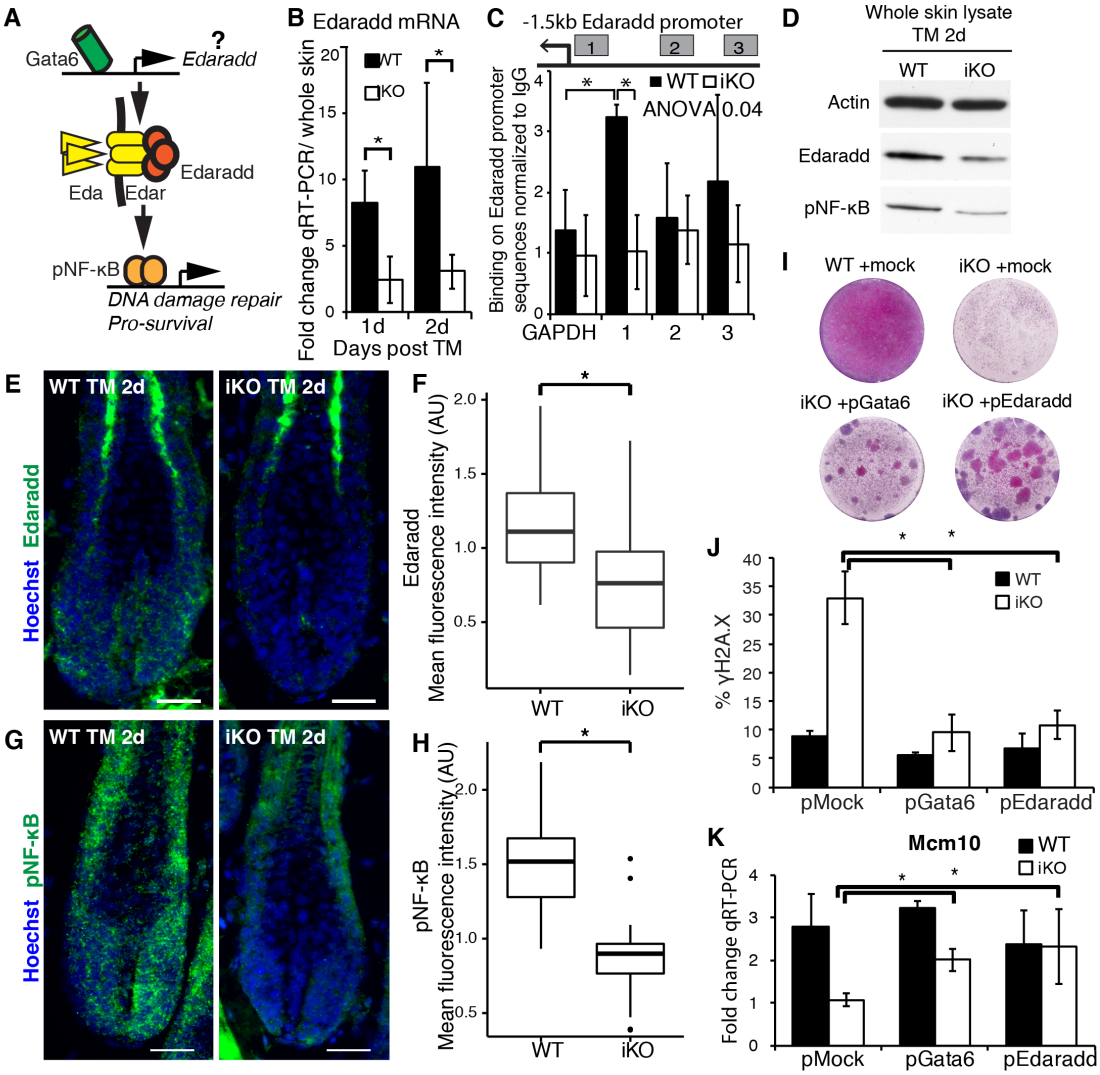
### *3.9 Gata6 augments Edaradd expression and NF-κB signaling in vivo*

The known interactions among *Eda*, *Edaradd* and NF-κB (Headon *et al*, 2001) and their implication in DNA damage, proliferation and apoptosis prompted us to further explore a potential upstream role of *Gata6* in regulating this pathway in the hair follicle (Fig 3.13A). To confirm that *Edaradd* is down-regulated by the *Gata6* iKO in the skin *in vivo*, we first performed qRT-PCR from total skin RNA. As seen previously in cultured keratinocytes (Fig 3.11B), *Edaradd* mRNA was significantly down-regulated in *Gata6* iKO skin compared to WT (Fig 3.13B). Western blotting showed a similar depletion of the EDARADD protein in *Gata6* iKO skin compared with WT and reduction of NF-κB protein phospho-p65 (Serine 536) (Fig 3.13D). Moreover, immunofluorescence staining and quantification confirmed reduced EDARADD protein and of NF-κB protein phospho-p65 (Serine 536) within the hair follicle matrix during anagen, in *Gata6* iKO skin (Fig 3.13E-H). Immunofluorescence signal was cytoplasmic as expected from this form of serine phosphorylated NF-κB (Sakurai *et al*, 2003; Moreno *et al*, 2010).

To test if GATA6 may directly bind to the *Edaradd* promoter to activate its transcription, we performed chromatin immunoprecipitation (ChIP) with a GATA6 specific antibody on chromatin prepared from *Gata6* WT and iKO cultured keratinocytes. The GATA6 bound chromatin was then analyzed by qPCR using primers for three regions within the *Edaradd* promoter (0.1kb, 1kb, and 1.5 kb

**Figure 3.13** *Edaradd*-NF-κB signaling are downstream of *Gata6*. (**A**) Model of potential *Gata6* role upstream of *Edaradd*/NF-κB. (**B**) qRT-PCR confirmation of the change in *Edaradd* mRNA expression *in-vivo* from mouse whole skin (average ± SD, 1d n=3, 2d n=4). *Unpaired t-test P-values: 1d = 0.02, 2d = 0.05. Mann-Whitney U test P-values: 1d=0.06, 2d=0.03. (**C**) ChIP-qPCR for GATA6 at three conserved binding sites within the *Edaradd* promoter. *GAPDH* served as a negative control. Binding site 1, 2, and 3 are located 50 bp, 1 kb, and 1.5 kb upstream of the transcription start site, respectively (average ± SD, n=4). *Unpaired t-test P-values *GAPDH*=0.4, 1=0.002, 2=0.8, 3=0.2. Mann-Whitney U test P-values: *GAPDH*=0.3, 1=0.06, 2=0.9, 3=0.2. (**D**) Western blot from whole skin lysate. Note decrease of *Edaradd* and activated pNF-κB in the iKO. (**E-F**) Staining for EDARADD and quantification show decrease in EDARADD protein levels in the matrix of *Gata6* iKO. *Unpaired t-test P-value = $3.5x10^{-14}$. n=100 follicles from 3 mice per genotype. (**G-H**) Staining for pNF-κB and quantification show decrease of active NF-κB in the *Gata6* iKO matrix. *Unpaired t-test P-value = $2.2x10^{-16}$. n=50 follicles from 3 mice. (**I**) Rescue of *Gata6* iKO keratinocyte growth by transfection with *pGata6* or *pEdaradd*. Cell plates of keratinocytes are stained with Rhodanile Blue (n=3 cell lines each). (**J**) Quantification of γH2A.X in cells stably transfected with *pMock*, *pGata6*, or *pEdaradd* plasmids, followed by induction with TM for 6 hours (average ± SD, n=4 with > 500 cells counted per condition). *Unpaired t-test P-values = $2x10^{-4}$ (iKO *pGata6* vs *pMock*), $2x10^{-4}$ (iKO *pEdaradd* vs *pMock*). (**K**) qRT-PCR analysis of

*Mcm10* expression in *pMock*, *pGata6*, and *pEdaradd* WT and iKO expressing cells (average ± SD, n=4). *Unpaired t-test P-values: $8x10^{-4}$ (iKO *pGata6* vs *pMock*), 0.03 (iKO *pEdaradd* vs *pMock*). Mann-Whitney U test P-values: 0.03 (iKO *pGata6* vs *pMock*), 0.03 (iKO *pEdaradd* vs *pMock*).

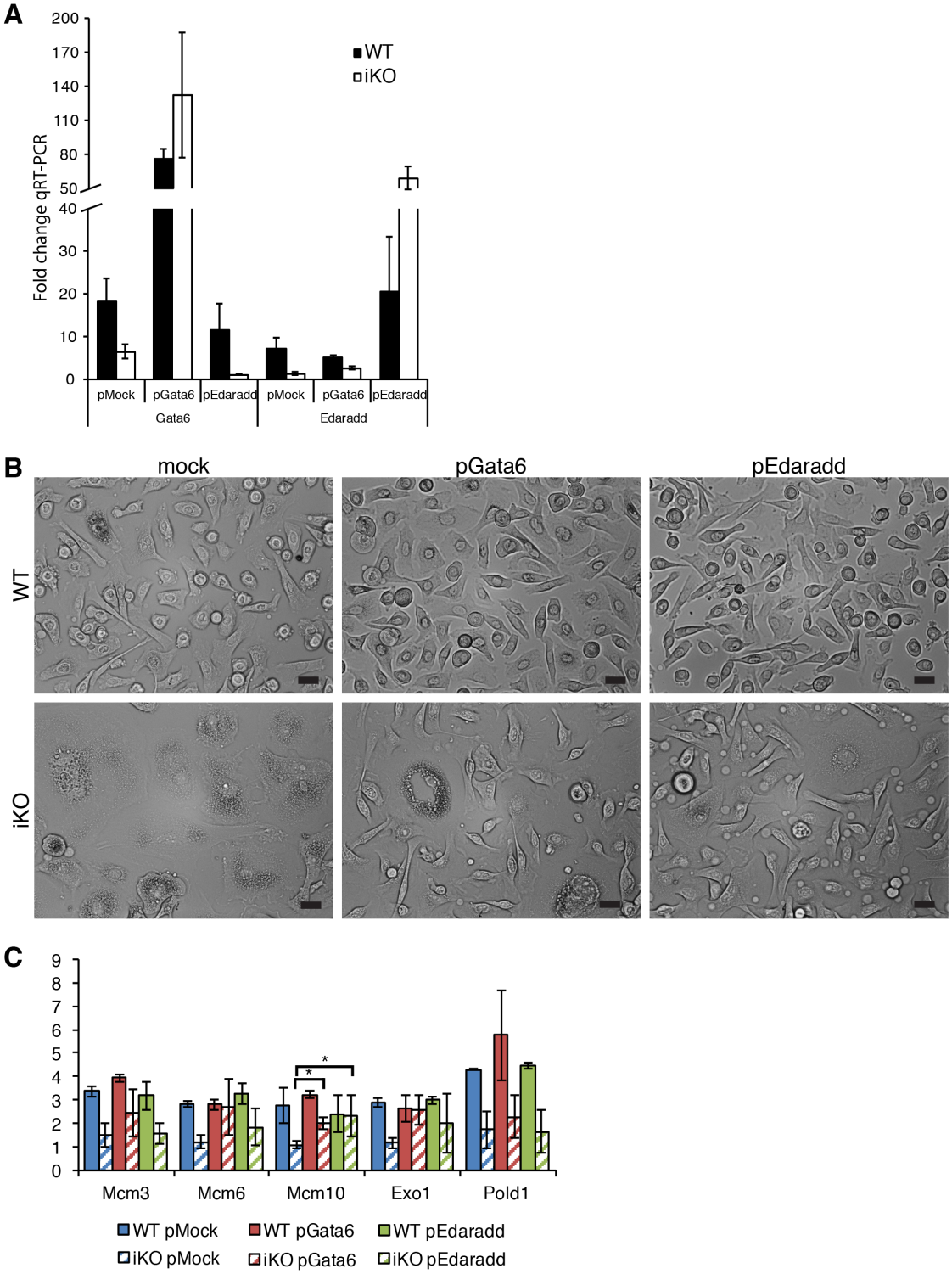

A
Gata6
?
Edaradd
Edaradd
Eda
Edar
pNF-κB
DNA damage repair
Pro-survival
B Edaradd mRNA
WT
KO
Fold change qRT-PCR/ whole skin
1d
2d
Days post TM
C -1.5kb Edaradd promoter
1
2
3
WT iKO
ANOVA 0.04
Binding on Edaradd promoter sequences normalized to IgG
GAPDH
D
Whole skin lysate
TM 2d
WT
iKO
Actin
Edaradd
pNF-κB
I
WT +mock
iKO +mock
iKO +pGata6
iKO +pEdaradd
E
WT TM 2d
iKO TM 2d
Hoechst Edaradd
F
Edaradd
Mean fluorescence intensity (AU)
WT
iKO
G
WT TM 2d
iKO TM 2d
Hoechst pNF-κB
H
pNF-κB
Mean fluorescence intensity (AU)
WT
iKO
J
% γH2A.X
WT
iKO
pMock
pGata6
pEdaradd
K
Mcm10
WT
iKO
Fold change qRT-PCR
pMock
pGata6
pEdaradd

upstream) that contained GATA6 binding sites conserved between mouse and human sequences (Fig 3.13C). We found significant enrichment over background at the 0.1 kb binding site, and also found that GATA6 binding was depleted from this site in iKO cells. Thus, we suggest GATA6 activates *Edaradd* transcription by directly binding its promoter although additional promoter bashing and mutagenesis assays will be required to firmly establish this direct link. Thus, our data show that GATA6 regulates *Edaradd* expression and NF-κB signaling *in vivo*, which may at least in part protect the hair follicle progenitor cells from DNA damage and premature apoptosis. We suggest that this may occur through direct binding of the *Edaradd* promoter, although additional mechanisms important in this regulation may not be excluded by our data.

### *3.10 Edaradd overexpression partially rescues Gata6 iKO keratinocytes*

To examine how important *Edaradd* levels are downstream of *Gata6* in cultured cells to control DNA damage, cell proliferation, and cell survival, we asked whether overexpression of *Edaradd* can rescue the *Gata6* iKO keratinocyte cultures. WT and iKO keratinocytes were stably transfected with either plasmid containing *Edaradd*, *Gata6* as a positive control, or the empty plasmid backbone as a negative control. Stable transfection of cells with *Gata6* or *Edaradd* plasmids resulted in overexpression of the respective genes (Fig 3.14A). After TM induction of *Gata6* iKO, unlike control-transfected cells that did not grow, both *Gata6* and *Edaradd* transfected cultures contained cells that survived *Gata6* iKO and continued to proliferate forming large colonies (Fig 3.13I and 3.14B). We also examined a potential

**Figure 3.14** Overexpression of *Edaradd* rescues *Gata6* iKO keratinocytes. (**A**) qRT-PCR analysis of *Gata6* and *Edaradd* in *Gata6* WT and iKO stably transfected with *pMock*, *pGata6*, or *pEdaradd*. (**B**) Phase contrast images of keratinocytes from cells stably transfected with empty plasmid, *pGata6*, or *pEdaradd*. Scale bars: 30 μm. (**C**) qRT-PCR of selected genes found to be overlapping between *Gata6* iKO and *Eda* transgenic analyzed in *Gata6* WT and iKO keratinocytes stably expressing *pMock*, *pGata6*, or *pEdaradd* (average ± SD, n=4). *Unpaired t-test P-values: $8x10^{-4}$ (iKO *pGata6* vs *pMock*), 0.03 (iKO *pEdaradd* vs *pMock*). Mann-Whitney U test P-values: 0.03 (iKO *pGata6* vs *pMock*), 0.03 (iKO *pEdaradd* vs *pMock*).


A
Fold change qRT-PCR
200
170
140
110
80
50
40
30
20
10
0
WT
iKO
pMock
pGata6
pEdaradd
pMock
pGata6
pEdaradd
Gata6
Edaradd
B
mock
pGata6
pEdaradd
WT
iKO
C
9
8
7
6
5
4
3
2
1
0
*
*
Mcm3
Mcm6
Mcm10
Exo1
Pold1
WT pMock
WT pGata6
WT pEdaradd
iKO pMock
iKO pGata6
iKO pEdaradd

direct link between *Edaradd* and the NF-κB activation downstream of *Gata6* in cultured keratinocytes, but results were variable and inconclusive (data not shown). As shown in the previous section this variability was not observed *in vivo* where NF-κB activity appeared consistently down-regulated upon *Gata6* loss (Fig. 3.13D-H). This discrepancy may indicate transient and compensatory effects on the activity of NF-κB *in vitro* due to cells being more dependent on this pathway in the stressful cell culture environment.

Analysis of DNA damage by γH2A.X immunofluorescence staining in the transfected cells shows that keratinocytes overexpressing either *Gata6* or *Edaradd* do not accumulate DNA damage in response to *Gata6* loss, demonstrating a rescue of this phenotype (Fig 3.13J). We next investigated whether this rescue of the DNA damage phenotype was related to the DNA replication and repair genes we found in common between *Gata6* and *Eda* expression datasets (Fig 3.14C). While *Mcm3*, *Mcm6*, and *Exo1* showed modest increases in expression in response to *Gata6* and *Edaradd* overexpression, *Mcm10* was significantly increased compared to mock transfected cells (Fig 3.13K). Interestingly, in addition to its role in DNA replication initiation (Homesley *et al*, 2000), *Mcm10* resists DNA damage and genomic instability by maintaining replication fork processivity and interacting with the RAD9-HUS1-RAD1 DNA repair complex (Chattopadhyay & Bielinsky, 2007; Becker *et al*, 2014; Miotto *et al*, 2014; Alver *et al*, 2014). This suggests that *Edaradd* plays an essential role downstream of *Gata6* in cultured keratinocytes, possibly through MCM10, to protect rapidly proliferating skin epithelial cells from cell death and to promote their survival and proliferation.

### *3.11 Discussion*

Here we implicate *Gata6*, a developmental transcription factor whose function was previously thought restricted to differentiation of endoderm and mesoderm-derived lineages (Molkentin, 2000; Maeda *et al*, 2005), in the regulation of adult homeostasis in an ectoderm-derived tissue. Specifically, we find that *Gata6* is expressed in the hair follicle's early progenitor cells (hair germ) at the transition between telogen (rest phase) and anagen (growth phase) and in the mature progenitor cells (matrix) at anagen. We show that *Gata6* plays essential roles in adult hair homeostasis (cycle) regulation at both phases, as follows. First, loss of *Gata6* from the epithelium prior to telogen/anagen transition blocks the progression of hair follicle into anagen by impairing activation and degeneration of early progenitor (hair germ cells). Second, loss of *Gata6* induced during anagen resulted in premature catagen followed by telogen, due to what appear to be defects in maintenance and self-renewal of the matrix progenitor cells. Since at least two transcription factors have already been implicated in control of early progenitor activation at the telogen/anagen transition (Lee *et al*, 2014; Osorio *et al*, 2008; 2011; Hoi *et al*, 2010; Sano *et al*, 1999; 2000), we focused our attention on the matrix maintenance phenotype during anagen, a process not currently well-understood (Fig 3.1A).

Hair follicle matrix cells have been deemed one of the most proliferative cell populations of any mammalian tissue (Lehrer *et al*, 1998) and must cope with high replication-associated DNA damage to ensure that genomic integrity is preserved. We find that *Gata6* is essential for the adult hair follicle progenitor (matrix) cells to self-

renew during the hair growth (anagen) phase while *Gata6* is dispensable for their terminal differentiation. *Gata6* loss resulted in rapid accumulation of DNA damage, impaired proliferation and apoptosis of the matrix progenitor cells. This coupled with continuous production of differentiation lineages (IRS, cortex, and lower ORS in the bulb) causes rapid exhaustion of the matrix progenitor cells and leads to an early dystrophic catagen, as previously reported in hair follicles severely damaged by chemotherapy and irradiation (Hendrix *et al*, 2005).

Based on genes changed in our RNA-seq analysis, we tested ROS as a possible source of DNA damage. While we did not find ROS levels significantly increased upon *Gata6* loss, it remains possible that impaired protection from oxidative stress contributes to the observed DNA damage phenotype. Importantly, we find that active passage through the cell cycle is deterministic of the level of DNA damage in *Gata6*-deficient cells and is increased in S-phase. This is consistent with replication-induced genotoxic stress, and is supported by misregulation of expression in a number of replication-associated genes upon *Gata6* loss. Most notable are the minichromosome maintenance proteins MCM3, MCM6, and MCM10, which are absolutely needed for DNA replication initiation, and even moderate decreases in the level of these proteins can cause genomic instability (Chuang *et al*, 2010).

Given the large number of genes that change expression upon *Gata6* loss, the mechanisms operating downstream are likely to be complex and involve additional direct and indirect target genes. A handful of genes we found changed have been previously implicated in hair follicle growth regulation *in vivo* (Headon *et al*, 2001; Shimomura *et al*, 2010; Hu *et al*, 2010b; Giacobbe *et al*, 2013; St-Pierre *et al*, 2006;

Shoag *et al*, 2013; Beaumont *et al*, 2011; Weiner *et al*, 2007). Of these, *Edaradd*, previously identified as a death domain adapter, is known to promote *Eda*/*Edar* signaling, which plays a well-established role in hair follicle morphogenesis and control of hair cycle, in anagen length and apoptosis during catagen (Mustonen *et al*, 2003; Cui & Schlessinger, 2006; Fessing *et al*, 2006). Furthermore, *Edaradd* is known to link *Edar* signaling to the NF-κB and JNK pathways (Headon *et al*, 2001; Yan *et al*, 2002; Kumar *et al*, 2001; Cui *et al*, 2002). Inhibition of NF-κB in the hair follicle is associated with increased apoptosis (Schmidt-Ullrich *et al*, 2001; Kloepper *et al*, 2014), as is also observed in *Edar* mutants (Fessing *et al*, 2006). The observed *Gata6* iKO phenotypes also resemble those of hair follicles from *Tak1*-deficient mice (an activator of NF-κB, as well as JNK and TGF-β/Smad pathways), which undergo G2/M cell cycle arrest, increased apoptosis, and premature catagen (Sayama *et al*, 2006; Omori *et al*, 2008; Sayama *et al*, 2010). Additionally, NF-κB has been shown to activate *Shh* expression in the hair matrix, which is associated with matrix cell proliferation (Hammerschmidt & Schlake, 2007; Xiong *et al*, 2013). Identification of GATA binding sites in the enhancer of the ligand *Eda* led to early speculation of a link (Pengue *et al*, 1999). In the hair follicle, we find that of 542 genes that change downstream of *Gata6*, 405 had conserved NF-κB binding sites; of these 397 also had GATA6 conserved binding sites suggesting possible co-regulation by *Gata6* and NF-κB. Here, we provide evidence that *Gata6* is essential for *Edaradd* expression and for constitutive activation of NF-κB in matrix cells in vivo during normal growth of the adult hair follicle. Our data in cell culture suggest that GATA6 may bind the *Edaradd* promoter, and that activation of *Edarrad* transcription may further control Mcm10

expression. Although loss of *Gata6* and *Edar* or NF-κB both display increased apoptosis, *Gata6* iKO hairs undergo a more robust degeneration compared to the premature catagen observed in the latter (Kloepper *et al*, 2014; Fessing *et al*, 2006). This discrepancy is likely due to the downregulation of other target genes and pathways that may also contribute to *Gata6*'s protective function (Fig 3.15).

Our study in the hair follicle may shed new light on how *Gata6* functions during tissue growth. As seen in studies of other tissues (Zhang *et al*, 2008), we observed that differentiated lineages are either not produced (telogen induction) or prematurely cease to be produced (anagen induction). However, taking advantage of the distinct compartmentalization of progenitor and differentiated cells and timing of events in the hair follicle, our study clearly demonstrates that terminal differentiation can occur in the absence of *Gata6* but that impairment in production of differentiated lineages is an indirect consequence of defective early progenitor cell activation (telogen induction) or self-renewal/genome maintenance and cell survival of mature progenitor cells (anagen induction). In light of our findings, it may be interesting to consider this alternative interpretation for the previously observed differentiation defects in other tissues.

In addition to its role in development, *Gata6* is frequently found to be amplified or overexpressed in various epithelial cancers such as colon (Shureiqi *et al*, 2007), pancreatic (Zhong *et al*, 2011), esophageal (Lin *et al*, 2012), and gastric carcinomas (Sulahian *et al*, 2013). Knockdown of *Gata6* in these cancer cells is associated with decreased proliferation and apoptosis, and moreover *Gata6* copy number is significantly correlated with patient survival

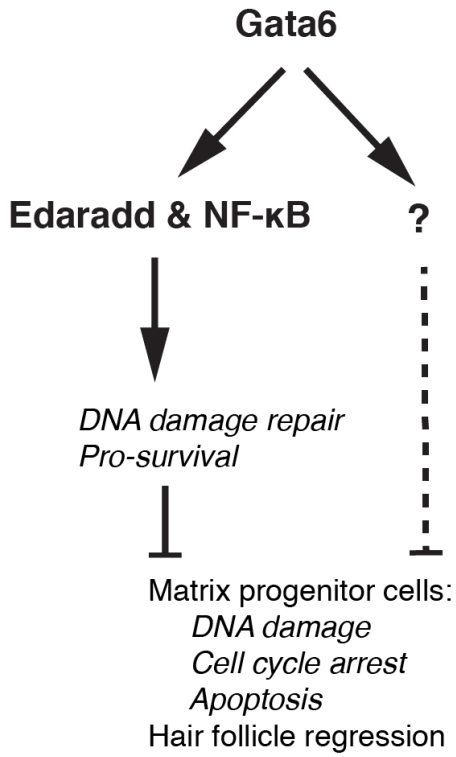



**Figure 3.15** Model for the role of *Gata6* in hair follicle progenitor cell survival through genome maintenance.

(Zhong *et al*, 2011; Lin *et al*, 2012; Shen *et al*, 2013). Our study points towards *Edaradd*-NF-κB as a potential mediator of *Gata6*'s survival abilities, and therefore a possible therapeutic target to be examined in the future for *Gata6* amplified cancers.

In conclusion, our current study adds to very recent publications (Lamm *et al*, 2016; Petroni *et al*, 2016) in suggesting that highly proliferative cells, such as the progenitor cells of the hair follicle and the brain, as well as embryonic stem cells employ developmentally controlled transcription factors (*i.e. SRF*, *MYCN*, and now *Gata6*) to protect against increased risk of genotoxic stress associated with rapid proliferation. Intriguingly, we find that MYCN binding sites were second most enriched (after NF-κB) on our list of transcription factors potentially associated with *Gata6* function (Table 3.2). We propose that proliferative cells cope with their increased vulnerability to replication-associated stress and genomic instability that may lead to cancer by employing developmentally regulated transcription factors. This occurs at critical stages to augment components of the DNA replication and DNA damage complexes, as a means to coordinate and sustain normal and rapid tissue growth.

### *3.12 Materials and methods*

*Mice*

For matrix lineage tracing, inducible multicolor reporter mice were generated by crossing *β-actin-CreER* mice with *R26R-Confetti* (Snippert *et al*, 2010). The *β-actin-CreER*$^{+}$*:Confetti*$^{+}$ progeny were induced with either a high dose of tamoxifen dissolved in corn oil (100 μg/g body weight; Sigma) or a low dose for inefficient

labeling and clonal lineage tracing (20 μg/g body weight). For *Gata6* iKO experiments, *Gata6*$^{fl/fl}$ mice (*Gata6*$^{tm2.1Sad}$; Jackson Laboratories) (Sodhi *et al*, 2006) were crossed with transgenic *K14-CreER*$^{T2}$ (Li *et al*, 2000). Mice were genotyped from tail snip DNA as described by Sodhi *et* al, 2006 (Fig 3.2D). *CreER*$^{T2}$ was activated by a single intraperitoneal injection of tamoxifen dissolved in corn oil (100 μg/g body weight). For BrdU experiments, mice were injected intraperitoneally with 50 μg/g body weight BrdU in PBS. All mouse work was approved by the Cornell University IACUC.

*Immunofluorescence staining, microscopy, and image processing*

Mouse back skin was embedded in optimal cutting temperature compound, frozen, cryosectioned, fixed, immunoblocked, and incubated with antibodies. Cultured keratinocytes were grown on glass coverslips, fixed, immunoblocked, and incubated with antibodies. Antibodies and dilutions used were: rat anti-BrdU (1:300; Abcam ab6326), rabbit anti-active Caspase-3 (1:500; R&D Systems AF835), mouse anti-AE13 (1:50; Immunoquest IQ292), mouse anti-AE15 (1:10; gift from T. T. Sun, New York University), rat anti-CD34 (1:50; BD Biosciences 553731), mouse anti-GATA3 (1:100; Santa Cruz Biotechnology sc-268), goat anti-GATA6 (1:200; R&D Systems AF1700), mouse anti-γH2A.X (1:200; Millipore 05-636), mouse anti-K14 (1:300; Abcam ab7800), rabbit anti-Edaradd (1:100; Abcam ab124484), and rabbit anti-NF-κB - p65 Ser536 (1:100; Cell Signaling 3033). When using mouse primary antibodies, endogenous mouse antigens were blocked using the M.O.M. basic kit (Vector Laboratories). Signal from Edaradd and NF-κB stainings were amplified using TSA

plus fluorescein system (PerkinElmer). Imaging was performed on a fluorescence light microscope (Nikon) with a Retiga EXi 12-bit CCD digital camera (QImaging) using the IP-Lab software (MVI). Images were assembled into montages in Photoshop (Adobe) and brightness, contrast, and levels were adjusted to the same extent for all samples within the same experiment.

*Cell culture*

Mouse keratinocytes were isolated from newborn epidermis, cultured in low-$Ca^{2+}$ keratinocyte E media (Tumbar, 2006), and were used between passage seven and ten. *K14-CreER$^{T2}$* was induced with 1 μM 4-hydroxytamoxifen. For comet assays, cells were analyzed under alkaline conditions by CometAssay Electrophoresis kit (Trevigen). For serum starvation experiment, serum levels were decreased over three days to maintain cells in quiescence without undergoing apoptosis according to the following protocol: cells grown to subconfluence in standard 15% serum media, 2 days in 7.5% serum media, 1 day in 3.75% serum media. Proliferation was assessed by EdU labeling and staining (Molecular Probes). Cell cycle analysis was conducted by labeling with BrdU Flow Kit (BD Biosciences) and analyzed on a BD LSRII Flow Cytometer. For rescue experiments, stably transfected cell lines were generated by co-transfection of pCMV-Tag2B-GATA6 (gift from E. Morrisey, University of Pennsylvania) or pSG5-HA-EDARADD (gift from D. Headon, University of Edinburgh) with empty backbone plasmid and TransFectin (Bio-Rad) followed by selection with G418 (Sigma). Stably transfected cells were subsequently treated with

tamoxifen for *Cre* induction of the *Gata6* iKO. Knockdown experiments were conducted with transfection of *Gata6* and scrambled shRNA vectors (OriGene).

*qRT-PCR*

Total RNA was isolated from cultured keratinocytes or mouse back skin using RNeasy Mini kit (Qiagen) and DNase treated (Ambion). All RNA samples were quality checked with an Agilent 2100 Bioanalyzer through the Cornell Biotechnology Resource Center. cDNA was synthesized using the iScript kit (Bio-Rad). qRT-PCR was conducted using homebrew SYBR Green PCR buffer and an iCycler PCR machine (Bio-Rad) was used to measure fluorescence. Primers used are listed in Table 3.4.

*RNA-seq*

Total RNA was prepared as described above. RNA-seq libraries were prepared using an Illumina TruSeq RNA Sample Prep Kit v2 then submitted to the Cornell Biotechnology Resource Center for sequencing with an Illumina HiSeq 2000. Differential expression of the RNA-seq data was performed using DEseq2 (Love *et al*, 2014). Gene ontology analyses were performed with DAVID (Huang *et al*, 2009a; 2009b). Enrichment of conserved transcription factor binding sites were performed with oPOSSUM (Ho Sui *et al*, 2005; 2007; Kwon *et al*, 2012), searching 10 kb upstream and downstream of the transcription start sites of differentially expressed genes for transcription factor binding motifs with a PhastCons conservation cutoff of 0.4 and comparing to the background database of all mouse genes. List of *Eda*

**Table 4** Primers used for qRT-PCR and ChIP-qPCR experiments.

| qRT-PCR and ChIP-qPCR Primers | | |
|---|---|---|
| Gene | Forward | Reverse |
| Gata6 | GTCCTGTGCCAACTGTCACAC | CTGCTGTTACCGGAGCAAGCT |
| Edaradd | TATCCTTTGCCATGTCAGAC | TGGAATTGACTGTATCACACTC |
| Apcdd1 | AAGCACCCCACATTCACCAT | CTCAGCCCCACACTCATTCC |
| Gls2 | CGGAAGTTAGACCCACGGAG | GGCTGTGCGGGAATCATAGT |
| Ppargc1b | AGATGGAACCCCAAGCGT | AGCACCTGGCACTCTACAAT |
| Rab17 | GGCCCTCCGGTACATGAAG | GGCACCCCTGAAGTAGAGGT |
| Foxn1 | GGGCTCACCTCACTATCCCTA | AGGATGCTGTAAGAGTAGATGGG |
| Edaradd ChIP 1 | CGGCTCCTAATCTCGTCCTC | TAGAAAGTGCTAGCGTGTGCG |
| Edaradd ChIP 2 | AAAGGTGGAGTTGCTGGTGG | GAGAGGCAGCTTAGCCTTGA |
| Edaradd ChIP 3 | AGGCTCAGGTAAGGTAGGGA | CGCTAACAAGACAGCCCAAC |

transgenic skin up-regulated genes were obtained from GEO GSE6952 (Cui *et al*, 2006). The RNA-seq data from this publication have been submitted to the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and will be available with the accession number GSE80354.

*ChIP*

Cells were fixed in 1% formaldehyde and sonicated with a Sonic Dismembrator 100 (Fisher) to obtain fragments between 300-600 bp. Immunoprecipitations were performed using rabbit anti-GATA6 antibody (H-92, Santa Cruz sc-9055), rabbit IgG (Cell Signaling Technology), and Protein A agarose beads (Millipore). Following immunoprecipitation, PCR purification kit (Qiagen) was used to purify DNA. ChIP signals were calculated by qPCR based on serial dilutions of input DNA standards. Primers were designed against regions in the *Edaradd* promoter that contained conserved GATA6 binding sites identified by Whole Genome rVista (Zambon *et al*, 2005). Primers used for ChIP-qPCR are listed in Table 3.4.

*Western Blot*

Total protein extracts from keratinocytes were subjected to SDS-PAGE and Western blot analysis. Blots were probed with mouse anti-actin (1:8000; Millipore MAB1501), rabbit anti-GATA6 (1:500; H-92, Santa Cruz sc-9055), rabbit anti-Edaradd (1:1000; Abcam ab124484), and rabbit anti-NF-κB - p65 Ser536 (1:1000; Cell Signaling 3033) antibodies.

*Statistical Analysis*

For statistical analysis we compare WT and iKO samples from $n > 3$ mice or independent cell lines per group by unpaired t-test. We assumed equal variance among the groups and p-values $\leq 0.05$ were considered significant. Since the small group size characteristic to biological samples does not allow accurate assessment of normal distribution of variances, p-values close to 0.05 in unpaired t-test indicate moderate proof of significance. To augment our statistical analyses, for some of our data we used a second, more conservative non-parametric test (e.g., Mann Whitney U test), which does not require assumption about normality or variances. These calculated p values are shown in figure legend.

# REFERENCES


Adams PD, Jasper H & Rudolph KL (2015) Aging-Induced Stem Cell Mutations as Drivers for Disease and Cancer. *Cell Stem Cell* **16:** 601–612

Alvarez S, Díaz M, Flach J, Rodriguez-Acebes S, López-Contreras AJ, Martínez D, Cañamero M, Fernández-Capetillo O, Isern J, Passegué E & Méndez J (2015) Replication stress caused by low MCM expression limits fetal erythropoiesis and hematopoietic stem cell functionality. **6:** 8548

Alver RC, Zhang T, Josephrajan A, Fultz BL, Hendrix CJ, Das-Bradoo S & Bielinsky A-K (2014) The N-terminus of Mcm10 is important for interaction with the 9-1-1 clamp and in resistance to DNA damage. *Nucleic Acids Res* **42:** 8389–8404

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

Beaumont KA, Hamilton NA, Moores MT, Brown DL, Ohbayashi N, Cairncross O, Cook AL, Smith AG, Misaki R, Fukuda M, Taguchi T, Sturm RA & Stow JL (2011) The recycling endosome protein Rab17 regulates melanocytic filopodia formation and melanosome trafficking. *Traffic* **12:** 627–643

Becker JR, Nguyen HD, Wang X & Bielinsky A-K (2014) Mcm10 deficiency causes defective-replisome-induced mutagenesis and a dependency on error-free postreplicative repair. *cc* **13:** 1737–1748

Berti M & Vindigni A (2016) Replication stress: getting back on track. *Nat Struct Mol Biol* **23:** 103–109

Beuling E, Aronson BE, Tran LMD, Stapleton KA, Horst Ter EN, Vissers LATM,

Verzi MP & Krasinski SD (2012) GATA6 Is Required for Proliferation, Migration, Secretory Cell Maturation, and Gene Expression in the Mature Mouse Colon. *Mol Cell Biol* **32:** 3392–3402

Beuling E, Baffour Awuah NYA, Stapleton KA, Aronson BE, Noah TK, Shroyer NF, Duncan SA, Fleet JC & Krasinski SD (2011) GATA Factors Regulate Proliferation, Differentiation, and Gene Expression in Small Intestine of Mature Mice. *Gastroenterology* **140:** 1219–1229.e2

Cai J, Lee J, Kopan R & Ma L (2009) Genetic interplays between Msx2 and Foxn1 are required for Notch1 expression and hair shaft differentiation. *Dev. Biol.* **326:** 420–430

Chattopadhyay S & Bielinsky A-K (2007) Human Mcm10 regulates the catalytic subunit of DNA polymerase-alpha and prevents DNA damage during replication. *Mol Biol Cell* **18:** 4085–4095

Chen C-C, Wang L, Plikus MV, Jiang TX, Murray PJ, Ramos R, Guerrero-Juarez CF, Hughes MW, Lee OK, Shi S, Widelitz RB, Lander AD & Chuong C-M (2015) Organ-Level Quorum Sensing Directs Regeneration in Hair Stem Cell Populations. *Cell* **161:** 277–290

Chen T, Heller E, Beronja S, Oshimori N, Stokes N & Fuchs E (2012) An RNA interference screen uncovers a new molecule in stem cell self-renewal and long-term regeneration. *Nature* **485:** 104–108

Choi YS, Zhang Y, Xu M, Yang Y, Ito M, Peng T, Cui Z, Nagy A, Hadjantonakis A-K, Lang RA, Cotsarelis G, Andl T, Morrisey EE & Millar SE (2013) Distinct functions for Wnt/β-catenin in hair follicle stem cell proliferation and survival and

interfollicular epidermal homeostasis. *Cell Stem Cell* **13:** 720–733

Chuang C-H, Wallace MD, Abratte C, Southard T & Schimenti JC (2010) Incremental Genetic Perturbations to MCM2-7 Expression and Subcellular Distribution Reveal Exquisite Sensitivity of Mice to DNA Replication Stress. *PLoS Genet* **6:** e1001110

Cui C-Y & Schlessinger D (2006) EDA signaling and skin appendage development. *cc* **5:** 2477–2483

Cui C-Y, Durmowicz M, Tanaka TS, Hartung AJ, Tezuka T, Hashimoto K, Ko MSH, Srivastava AK & Schlessinger D (2002) EDA targets revealed by skin gene expression profiles of wild-type, Tabby and Tabby EDA-A1 transgenic mice. *Hum. Mol. Genet.* **11:** 1763–1773

Cui C-Y, Hashimoto T, Grivennikov SI, Piao Y, Nedospasov SA & Schlessinger D (2006) Ectodysplasin regulates the lymphotoxin-beta pathway for hair differentiation. *Proc Natl Acad Sci USA* **103:** 9142–9147

DasGupta R & Fuchs E (1999) Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126:** 4557–4568

Ellis T, Gambardella L, Horcher M, Tschanz S, Capol J, Bertram P, Jochum W, Barrandon Y & Busslinger M (2001) The transcriptional repressor CDP (Cutl1) is essential for epithelial cell differentiation of the lung and the hair follicle. *Genes & Development* **15:** 2307–2319

Fessing MY, Sharova TY, Sharov AA, Atoyan R & Botchkarev VA (2006) Involvement of the Edar signaling in the control of hair follicle involution

(catagen). *Am. J. Pathol.* **169:** 2075–2084

Flach J, Bakker ST, Mohrin M, Conroy PC, Pietras EM, Reynaud D, Alvarez S, Diolaiti ME, Ugarte F, Forsberg EC, Le Beau MM, Stohr BA, Méndez J, Morrison CG & Passegué E (2014) Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature*

Folgueras AR, Guo X, Pasolli HA, Stokes N, Polak L, Zheng D & Fuchs E (2013) Architectural niche organization by LHX2 is linked to hair follicle stem cell function. *Cell Stem Cell* **13:** 314–327

Fuchs E & Chen T (2013) A matter of life and death: self-renewal in stem cells. *EMBO Rep.* **14:** 39–48

Giacobbe A, Bongiorno-Borbone L, Bernassola F, Terrinoni A, Markert EK, Levine AJ, Feng Z, Agostini M, Zolla L, Agrò AF, Notterman DA, Melino G & Peschiaroli A (2013) p63 regulates glutaminase 2 expression. *cc* **12:** 1395–1405

Hammerschmidt B & Schlake T (2007) Localization of Shh expression by Wnt and Eda affects axial polarity and shape of hairs. *Dev. Biol.* **305:** 246–261

Headon DJ, Emmal SA, Ferguson BM, Tucker AS, Justice MJ, Sharpe PT, Zonana J & Overbeek PA (2001) Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature* **414:** 913–916

Hendrix S, Handjiski B, Peters EMJ & Paus R (2005) A guide to assessing damage response pathways of the hair follicle: lessons from cyclophosphamide-induced alopecia in mice. *J Invest Dermatol* **125:** 42–51

Ho Sui SJ, Fulton DL, Arenillas DJ, Kwon AT & Wasserman WW (2007) oPOSSUM: integrated tools for analysis of regulatory motif over-representation. *Nucleic Acids*

*Res* **35:** W245–52

Ho Sui SJ, Mortimer JR, Arenillas DJ, Brumm J, Walsh CJ, Kennedy BP & Wasserman WW (2005) oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. *Nucleic Acids Res* **33:** 3154–3164

Hoi CSL, Lee SE, Lu S-Y, McDermitt DJ, Osorio KM, Piskun CM, Peters RM, Paus R & Tumbar T (2010) Runx1 directly promotes proliferation of hair follicle stem cells and epithelial tumor formation in mouse skin. *Mol Cell Biol* **30:** 2518–2536

Homesley L, Lei M, Kawasaki Y, Sawyer S, Christensen T & Tye BK (2000) Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. *Genes & Development* **14:** 913–926

Horsley V, Aliprantis AO, Polak L, Glimcher LH & Fuchs E (2008) NFATc1 balances quiescence and proliferation of skin stem cells. *Cell* **132:** 299–310

Hsu Y-C, Li L & Fuchs E (2014) Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. *Cell* **157:** 935–949

Hu B, Lefort K, Qiu W, Nguyen B-C, Rajaram RD, Castillo E, He F, Chen Y, Angel P, Brisken C & Dotto GP (2010a) Control of hair follicle cell fate by underlying mesenchyme through a CSL-Wnt5a-FoxN1 regulatory axis. *Genes & Development* **24:** 1519–1532

Hu W, Zhang C, Wu R, Sun Y, Levine A & Feng Z (2010b) Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. *Proc Natl Acad Sci USA* **107:** 7455–7460

Huang DW, Huang DW, Sherman BT, Sherman BT, Lempicki RA & Lempicki RA

(2009a) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4:** 44–57

Huang DW, Sherman BT & Lempicki RA (2009b) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37:** 1–13

Hwang J, Mehrani T, Millar SE & Morasso MI (2008) Dlx3 is a crucial regulator of hair follicle differentiation and cycling. *Development* **135:** 3149–3159

Janssens S & Tschopp J (2006) Signals from within: the DNA-damage-induced NF-kappaB response. *Cell Death Differ.* **13:** 773–784

Jave-Suarez LF, Winter H, Langbein L, Rogers MA & Schweizer J (2002) HOXC13 is involved in the regulation of human hair keratin gene expression. *J Biol Chem* **277:** 3718–3726

Kadaja M, Keyes BE, Lin M, Pasolli HA, Genander M, Polak L, Stokes N, Zheng D & Fuchs E (2014) SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. *Genes & Development* **28:** 328–341

Kaufman CK, Zhou P, Pasolli HA, Rendl M, Bolotin D, Lim K-C, Dai X, Alegre M-L & Fuchs E (2003) GATA-3: an unexpected regulator of cell lineage determination in skin. *Genes & Development* **17:** 2108–2122

Kim B-K & Yoon SK (2013) Hairless down-regulates expression of Msx2 and its related target genes in hair follicles. *J. Dermatol. Sci.* **71:** 203–209

Kloepper JE, Ernst N, Ernst N, Krieger K, Krieger K, Bodó E, Bodó E, Bíró T, Bíró T, Haslam IS, Haslam IS, Schmidt-Ullrich R & Paus R (2014) NF-κB activity is required for anagen maintenance in human hair follicles in vitro. *J Investig*

*Dermatol* **134:** 2036–2038

Kosan C & Godmann M (2016) Genetic and Epigenetic Mechanisms That Maintain Hematopoietic Stem Cell Function. *Stem Cells Int* **2016:** 5178965

Kumar A, Eby MT, Sinha S, Jasmin A & Chaudhary PM (2001) The ectodermal dysplasia receptor activates the nuclear factor-kappaB, JNK, and cell death pathways and binds to ectodysplasin A. *J Biol Chem* **276:** 2668–2677

Kwei KA, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi Y-L, Chatterjee S, Karikari CA, Salari K, Wang P, Hernandez-Boussard T, Swarnalata G, van de Rijn M, Maitra A & Pollack JR (2008) Genomic Profiling Identifies GATA6 as a Candidate Oncogene Amplified in Pancreatobiliary Cancer. *PLoS Genet* **4:** e1000081

Kwon AT, Arenillas DJ, Worsley Hunt R & Wasserman WW (2012) oPOSSUM-3: advanced analysis of regulatory motif over-representation across genes or ChIP-Seq datasets. *G3 (Bethesda)* **2:** 987–1002

Lamm N, Ben-David U, Golan-Lev T, Storchová Z, Benvenisty N & Kerem B (2016) Genomic Instability in Human Pluripotent Stem Cells Arises from Replicative Stress and Chromosome Condensation Defects. *Cell Stem Cell* **18:** 253–261

Lay K, Kume T & Fuchs E (2016) FOXC1 maintains the hair follicle stem cell niche and governs stem cell quiescence to preserve long-term tissue-regenerating potential. *Proc Natl Acad Sci USA* **113:** E1506–15

Lee J, Hoi CSL, Lilja KC, White BS, Lee SE, Shalloway D & Tumbar T (2013) Runx1 and p21 synergistically limit the extent of hair follicle stem cell quiescence in vivo. *Proc Natl Acad Sci USA* **110:** 4634–4639

Lee SE, Sada A, Zhang M, McDermitt DJ, Lu SY, Kemphues KJ & Tumbar T (2014) High Runx1 Levels Promote a Reversible, More-Differentiated Cell State in Hair-Follicle Stem Cells during Quiescence. *Cell Rep* **6:** 499–513

Legué E & Nicolas J-F (2005) Hair follicle renewal: organization of stem cells in the matrix and the role of stereotyped lineages and behaviors. *Development* **132:** 4143–4154

Legué E, Sequeira I & Nicolas J-F (2010) Hair follicle renewal: authentic morphogenesis that depends on a complex progression of stem cell lineages. *Development* **137:** 569–577

Lehrer MS, Sun TT & Lavker RM (1998) Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *Journal of Cell Science* **111 ( Pt 19):** 2867–2875

Li M, Indra AK, Warot X, Brocard J, Messaddeq N, Kato S, Metzger D & Chambon P (2000) Skin abnormalities generated by temporally controlled RXRalpha mutations in mouse epidermis. *Nature* **407:** 633–636

Lien W-H, Polak L, Lin M, Lay K, Zheng D & Fuchs E (2014) In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. *Nat Cell Biol* **16:** 179–190

Lin L, Bass AJ, Lockwood WW, Wang Z, Silvers AL, Thomas DG, Chang AC, Lin J, Orringer MB, Li W, Glover TW, Giordano TJ, Lam WL, Meyerson M & Beer DG (2012) Activation of GATA binding protein 6 (GATA6) sustains oncogenic lineage-survival in esophageal adenocarcinoma. *Proc Natl Acad Sci USA* **109:** 4251–4256

Love MI, Huber W & Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15:** 550

Lowry WE, Blanpain C, Nowak JA, Guasch G, Lewis L & Fuchs E (2005) Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. *Genes & Development* **19:** 1596–1611

Ma L, Liu J, Wu T, Plikus M, Jiang TX, Bi Q, Liu Y-H, Müller-Röver S, Peters H, Sundberg JP, Maxson R, Maas RL & Chuong C-M (2003) 'Cyclic alopecia' in Msx2 mutants: defects in hair cycling and hair shaft differentiation. *Development* **130:** 379–389

Maeda M, Ohashi K & Ohashi-Kobayashi A (2005) Further extension of mammalian GATA-6. *Dev. Growth Differ.* **47:** 591–600

Merrill BJ, Gat U, DasGupta R & Fuchs E (2001) Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. *Genes & Development* **15:** 1688–1705

Miotto B, Chibi M, Xie P, Koundrioukoff S, Moolman-Smook H, Pugh D, Debatisse M, He F, Zhang L & Defossez P-A (2014) The RBBP6/ZBTB38/MCM10 Axis Regulates DNA Replication and Common Fragile Site Stability. *Cell Rep* **7:** 575–587

Molkentin JD (2000) The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem* **275:** 38949–38952

Moreno R, Sobotzik J-M, Schultz C & Schmitz ML (2010) Specification of the NF-kappaB transcriptional response by p65 phosphorylation and TNF-induced nuclear

translocation of IKK epsilon. *Nucleic Acids Res* **38:** 6029–6044

Morgner J, Ghatak S, Jakobi T, Dieterich C, Aumailley M & Wickström SA (2015) Integrin-linked kinase regulates the niche of quiescent epidermal stem cells. **6:** 8198

Morrisey EE, Tang Z, Sigrist K, Lu MM, Jiang F, Ip HS & Parmacek MS (1998) GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes & Development* **12:** 3579–3590

Mustonen T, Pispa J, Mikkola ML, Pummila M, Kangas AT, Pakkasjärvi L, Jaatinen R & Thesleff I (2003) Stimulation of ectodermal organ development by Ectodysplasin-A1. *Dev. Biol.* **259:** 123–136

Omori E, Morioka S, Matsumoto K & Ninomiya-Tsuji J (2008) TAK1 regulates reactive oxygen species and cell death in keratinocytes, which is essential for skin integrity. *J Biol Chem* **283:** 26161–26168

Oro AE & Higgins K (2003) Hair cycle regulation of Hedgehog signal reception. *Dev. Biol.* **255:** 238–248

Osorio KM, Lee SE, McDermitt DJ, Waghmare SK, Zhang YV, Woo HN & Tumbar T (2008) Runx1 modulates developmental, but not injury-driven, hair follicle stem cell activation. *Development* **135:** 1059–1068

Osorio KM, Lilja KC & Tumbar T (2011) Runx1 modulates adult hair follicle stem cell emergence and maintenance from distinct embryonic skin compartments. *J Cell Biol* **193:** 235–250

Pengue G, Srivastava AK, Kere J, Schlessinger D & Durmowicz MC (1999) Functional characterization of the promoter of the X-linked ectodermal dysplasia

gene. *J Biol Chem* **274:** 26477–26484

Petroni M, Sardina F, Heil C, Sahún-Roncero M, Colicchia V, Veschi V, Albini S, Fruci D, Ricci B, Soriani A, Di Marcotullio L, Screpanti I, Gulino A & Giannini G (2016) The MRN complex is transcriptionally regulated by MYCN during neural cell proliferation to control replication stress. *Cell Death Differ.* **23:** 197–206

Sada A & Tumbar T (2013) New insights into mechanisms of stem cell daughter fate determination in regenerative tissues. *Int Rev Cell Mol Biol* **300:** 1–50

Sakurai H, Suzuki S, Kawasaki N, Nakano H, Okazaki T, Chino A, Doi T & Saiki I (2003) Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappaB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J Biol Chem* **278:** 36916–36923

Sano S, Itami S, Takeda K, Tarutani M, Yamaguchi Y, Miura H, Yoshikawa K, Akira S & Takeda J (1999) Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO J* **18:** 4657–4668

Sano S, Kira M, Takagi S, Yoshikawa K, Takeda J & Itami S (2000) Two distinct signaling pathways in hair cycle induction: Stat3-dependent and -independent pathways. *Proc Natl Acad Sci USA* **97:** 13824–13829

Sayama K, Hanakawa Y, Hanakawa Y, Nagai H, Nagai H, Shirakata Y, Shirakata Y, Dai X, Dai X, Hirakawa S, Hirakawa S, Tokumaru S, Tokumaru S, Tohyama M, Tohyama M, Yang L, Yang L, Sato S, Sato S, Shizuo A, et al (2006) Transforming growth factor-beta-activated kinase 1 is essential for differentiation and the prevention of apoptosis in epidermis. *J Biol Chem* **281:** 22013–22020

Sayama K, Kajiya K, Sugawara K, Sato S, Hirakawa S, Shirakata Y, Hanakawa Y, Dai X, Ishimatsu-Tsuji Y, Metzger D, Chambon P, Akira S, Paus R, Kishimoto J & Hashimoto K (2010) Inflammatory mediator TAK1 regulates hair follicle morphogenesis and anagen induction shown by using keratinocyte-specific TAK1-deficient mice. *PLoS ONE* **5:** e11275

Schmidt-Ullrich R, Aebischer T, Hülsken J, Birchmeier W, Klemm U & Scheidereit C (2001) Requirement of NF-kappaB/Rel for the development of hair follicles and other epidermal appendices. *Development* **128:** 3843–3853

Shen F, Li J, Cai W, Zhu G, Gu W, Jia L & Xu B (2013) GATA6 predicts prognosis and hepatic metastasis of colorectal cancer. *Oncol. Rep.* **30:** 1355–1361

Shimomura Y, Agalliu D, Vonica A, Luria V, Wajid M, Baumer A, Belli S, Petukhova L, Schinzel A, Brivanlou AH, Barres BA & Christiano AM (2010) APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. *Nature* **464:** 1043–1047

Shoag J, Haq R, Zhang M, Liu L, Rowe GC, Jiang A, Koulisis N, Farrel C, Amos CI, Wei Q, Lee JE, Zhang J, Kupper TS, Qureshi AA, Cui R, Han J, Fisher DE & Arany Z (2013) PGC-1 Coactivators Regulate MITF and the Tanning Response. *Mol Cell* **49:** 145–157

Shureiqi I, Zuo X, Broaddus R, Wu Y, Guan B, Morris JS & Lippman SM (2007) The transcription factor GATA-6 is overexpressed in vivo and contributes to silencing 15-LOX-1 in vitro in human colon cancer. *FASEB J.* **21:** 743–753

Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD & Clevers H (2010) Intestinal

crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* **143:** 134–144

Sodhi CP, Li J & Duncan SA (2006) Generation of mice harbouring a conditional loss-of-function allele of Gata6. *BMC Dev. Biol.* **6:** 19

Sotiropoulou PA, Sotiropoulou PA, Karambelas AE, Debaugnies M, Candi A, Bouwman P, Moers V, Revenco T, Rocha AS, Sekiguchi K, Jonkers J & Blanpain C (2012) BRCA1 deficiency in skin epidermis leads to selective loss of hair follicle stem cells and their progeny. *Genes & Development* **27:** 39–51

St-Jacques B, Dassule HR, Karavanova I, Botchkarev VA, Li J, Danielian PS, McMahon JA, Lewis PM, Paus R & McMahon AP (1998) Sonic hedgehog signaling is essential for hair development. *Curr Biol* **8:** 1058–1068

St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, Handschin C, Zheng K, Lin JD, Yang W, Simon DK, Bachoo R & Spiegelman BM (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* **127:** 397–408

Sulahian R, Casey F, Shen J, Qian ZR, Shin H, Ogino S, Weir BA, Vazquez F, Liu XS, Hahn WC, Bass AJ, Chan V & Shivdasani RA (2013) An integrative analysis reveals functional targets of GATA6 transcriptional regulation in gastric cancer. *Oncogene*

Tong X & Coulombe PA (2006) Keratin 17 modulates hair follicle cycling in a TNFalpha-dependent fashion. *Genes & Development* **20:** 1353–1364

Tumbar T (2006) Epithelial skin stem cells. *Meth Enzymol* **419:** 73–99

Wang L, Siegenthaler JA, Dowell RD & Yi R (2016) Foxc1 reinforces quiescence in

self-renewing hair follicle stem cells. *Science* **351:** 613–617

Wang LC, Liu ZY, Gambardella L, Delacour A, Shapiro R, Yang J, Sizing I, Rayhorn P, Garber EA, Benjamin CD, Williams KP, Taylor FR, Barrandon Y, Ling L & Burkly LC (2000) Conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration. *J Invest Dermatol* **114:** 901–908

Weiner L, Han R, Scicchitano BM, Li J, Hasegawa K, Grossi M, Lee D & Brissette JL (2007) Dedicated epithelial recipient cells determine pigmentation patterns. *Cell* **130:** 932–942

Weissbein U, Benvenisty N & Ben-David U (2014) Quality control: Genome maintenance in pluripotent stem cells. *J Cell Biol* **204:** 153–163

Whissell G, Montagni E, Martinelli P, Hernando-Momblona X, Sevillano M, Jung P, Cortina C, Calon A, Abuli A, Castells A, Castellvi-Bel S, Nacht AS, Sancho E, Stephan-Otto Attolini C, Vicent GP, Real FX & Batlle E (2014) The transcription factor GATA6 enables self-renewal of colon adenoma stem cells by repressing BMP gene expression. *Nat Cell Biol*

Xiong Y, Li W, Shang C, Chen RM, Han P, Yang J, Stankunas K, Wu B, Pan M, Zhou B, Longaker MT & Chang C-P (2013) Brg1 governs a positive feedback circuit in the hair follicle for tissue regeneration and repair. *Dev Cell* **25:** 169–181

Yan M, Zhang Z, Brady JR, Schilbach S, Fairbrother WJ & Dixit VM (2002) Identification of a novel death domain-containing adaptor molecule for ectodysplasin-A receptor that is mutated in crinkled mice. *Curr Biol* **12:** 409–413

Yang H, Lu MM, Zhang L, Whitsett JA & Morrisey EE (2002) GATA6 regulates

differentiation of distal lung epithelium. *Development* **129:** 2233–2246

Zambon AC, Zhang L, Minovitsky S, Kanter JR, Prabhakar S, Salomonis N, Vranizan K, Dubchak I, Conklin BR & Insel PA (2005) Gene expression patterns define key transcriptional events in cell-cycle regulation by cAMP and protein kinase A. *Proc Natl Acad Sci USA* **102:** 8561–8566

Zhang Y, Goss AM, Cohen ED, Kadzik R, Lepore JJ, Muthukumaraswamy K, Yang J, DeMayo FJ, Whitsett JA, Parmacek MS & Morrisey EE (2008) A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. *Nat Genet* **40:** 862–870

Zhang YV, Cheong J, Ciapurin N, McDermitt DJ & Tumbar T (2009) Distinct self-renewal and differentiation phases in the niche of infrequently dividing hair follicle stem cells. *Cell Stem Cell* **5:** 267–278

Zhao R, Watt AJ, Battle MA, Li J, Bondow BJ & Duncan SA (2008) Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. *Dev. Biol.* **317:** 614–619

Zhao R, Watt AJ, Li J, Luebke-Wheeler J, Morrisey EE & Duncan SA (2005) GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. *Mol Cell Biol* **25:** 2622–2631

Zhong Y, Wang Z, Fu B, Pan F, Yachida S, Dhara M, Albesiano E, Li L, Naito Y, Vilardell F, Cummings C, Martinelli P, Li A, Yonescu R, Ma Q, Griffin CA, Real FX & Iacobuzio-Donahue CA (2011) GATA6 Activates Wnt Signaling in Pancreatic Cancer by Negatively Regulating the Wnt Antagonist Dickkopf-1. *PLoS ONE* **6:** e22129

Zhou P, Byrne C, Jacobs J & Fuchs E (1995) Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes & Development* **9:** 700–713

# CHAPTER 4

# CONCLUSIONS AND FUTURE DIRECTIONS

## *4.1 Genome maintenance of stem and progenitor cells*

The work described in the previous chapter highlights a problem faced by highly proliferative cells such as progenitor cells, embryonic stem cells, and continuously dividing stem cells such as those of the intestine and epidermis. While many tissue stem cells are protected from replication stress by remaining largely quiescent through their lifetime, their progenitor cells, such as the hair follicle's matrix cells, assume the burden of proliferating to maintain their resident tissues and thus face the risk of damage to their genome as a result of their highly proliferative status. The work presented in Chapter 3 on hair follicle matrix cells, along with other recent publications regarding neural progenitor cells and embryonic stem cells (Petroni *et al*, 2016; Lamm *et al*, 2016), suggest that developmental transcription factors such as *Gata6*, *MYCN*, and *SRF* transcriptionally reinforce protective mechanisms critical for the cells to resist replication-induced damage to their genomes.

Stem and progenitor cell maintenance is critical for proper tissue homeostasis, and genomic damage to these cells is implicated in aging and cancer initiation (Insinga *et al*, 2014). Stem cells and progenitor cells have been found to have distinct responses to DNA damage. In response to ionizing radiation induced double-strand breaks, hematopoietic, mammary, and hair follicle stem cells (HFSCs) have been shown to inhibit tumor suppressor *p53* activation and apoptosis, instead allowing the breaks to

be repaired through non-homologous end joining (NHEJ) (Cicalese *et al*, 2009; Insinga *et al*, 2013; Sotiropoulou *et al*, 2010). In the HFSCs in particular, the inhibition of apoptosis has been shown to be due to both the rapid downregulation of *p53* and upregulation of the anti-apoptotic gene *Bcl-2* (Sotiropoulou *et al*, 2010). Additionally, HFSCs accelerates the activity of NHEJ by increasing the nuclear localization of the critical NHEJ protein DNA-PK. This response to DNA damage in relatively quiescent tissue stem cells may have evolved in order to preserve the limited pool of stem cells, as apoptosis would deplete the stem cell pool leading to premature aging phenotypes (Behrens *et al*, 2014). Interestingly, tissue stem cell response to DNA damage differs in other tissues. Melanocyte stem cells, also found in the bulge niche of the hair follicle, undergo differentiation after irradiation leading to premature depletion of the stem cell pool and graying of the hair (Inomata *et al*, 2009). The two stem cell populations of the intestine also display distinct DNA damage responses. The less active stem cells at the +4 position of the intestinal crypt readily initiate apoptosis in response to even low doses of irradiation, whereas their neighboring actively cycling $Lgr5^{+}$ population of intestinal stem cells are relatively resistant to irradiation and are able to repair damage by homologous recombination (Barker *et al*, 2007; Hua *et al*, 2012).

Despite their importance to tissue homeostasis and high risk of DNA damage due to proliferation, relatively little is known about how progenitor cells respond to DNA damage. Multipotent progenitors and common myeloid progenitors of the hematopoietic system undergo *p53*-dependent apoptosis following irradiation (Insinga *et al*, 2013). In the hair follicle, matrix progenitor cells have been shown to depend on

expression of the DNA damage response factor *BRCA1* for survival during normal homeostasis (Sotiropoulou *et al*, 2012). Similar to what I showed in Chapter 3 in response to *Gata6* loss, conditional ablation of *BRCA1* in the skin epithelium causes matrix progenitor cells to accumulate double-strand breaks and undergo apoptosis. This, along with my work, suggest that hair follicle progenitor cells are under significant genotoxic stress, likely from rapid continuous proliferation, and require reinforced DNA repair and survival mechanisms such as by *BRCA1* and *Gata6* activated NF-κB, and possibly other factors to maintain their genomes and sustain growth.

Although stem cell defects are often considered a more significant risk in terms of tissue aging and tumor initiation due to their long lifespan, defects in progenitor cells are also of great consequence for tissue homeostasis. As demonstrated by work in the hair follicle, loss of progenitor cells in turn causes abnormal sustained activation of the stem cell population, leading to hair loss due to premature exhaustion of the stem cell pool (Sotiropoulou *et al*, 2012). Additionally, more thorough understanding of progenitor cells may uncover mechanisms of significance to other highly proliferative cells such as embryonic stem cells and tumor cells.

### *4.2 Expanding the role of Gata6*

*Gata6* has predominantly been known as a transcription factor involved in embryonic differentiation and endodermal and mesodermal tissue development (Lentjes *et al*, 2016). Recent work in the lung (Zhang *et al*, 2008), as well as the work presented here in the hair follicle, show that in addition to its developmental functions

(summarized in Chapter 1.4), *Gata6* also has a critical role in tissue homeostasis through stem and progenitor cell regulation. In the lung, loss of *Gata6* in the airway epithelium causes a loss of the terminally differentiated cells of the bronchiolar and alveolar lineages, with a concomitant expansion of the bronchioalveolar stem cells (BASCs - a regional progenitor population of the lungs) (Zhang *et al*, 2008). This increased proliferation and decreased differentiation of the BASCs in *Gata6* ablated cells was found to be associated with increased Wnt–β-catenin signaling due to downregulation of the Wnt antagonist *Fzd2*.

Interestingly, although *Gata6* functions in progenitor cell populations in both the lung and hair follicle, it appears to have divergent functions in the two tissues. Whereas in the lung *Gata6* appears to promote differentiation and limits self-renewal, in the hair follicle *Gata6* maintains self-renewal and does not seem to have a direct effect on differentiation (although differentiated cells are eventually lost due to depletion of progenitor cells from apoptosis). While the function of *Gata6* may be tissue specific, it would be valuable to reevaluate the effects of *Gata6* depletion in the lung with regard to its effects on DNA damage and survival. Unlike the simplistic organization of the hair follicle, the stem, progenitor, and differentiated cells of the lung are not compartmentalized in distinct regions, and the lung has been found to contain multiple stem and progenitor populations. It may be that *Gata6* in the lung actually functions in maintaining another progenitor population downstream of the BASCs in a manner similar to the hair matrix cells, explaining the inconsistency between the results of the two tissues (Hogan *et al*, 2014).

In addition to expanding *Gata6*'s function to a new ectodermal tissue, the hair follicle, this work provides a valuable link connecting *Gata6* to the NF-κB pathway for genomic maintenance of hair follicle progenitor cells. The NF-κB pathway is generally most recognized for its well-established role in regulating proliferation and apoptosis in inflammation and stress responses (Janssens & Tschopp, 2006). It is well-known for its involvement in chemotherapy resistance by modulating the response to DNA-damage to promote cell survival by activating cell cycle checkpoints and the DNA repair machinery (Janssens & Tschopp, 2006). The NF-κB pathway has been implicated in other developmental processes besides the hair follicle cycle, such as cell survival of the embryonic liver and neuronal epithelium, limb and skeletal development, and inhibition of epidermal differentiation (Li *et al*, 1999b; 1999a; Rudolph *et al*, 2000; Takeda *et al*, 1999; Hu *et al*, 1999; Li *et al*, 2000). NF-κB also protects adult hematopoietic progenitor cells as well as embryonic and adult neurons against apoptosis (Turco *et al*, 2004; Mizrahi *et al*, 2014; Bhakar *et al*, 2002; Lezoualc'h *et al*, 1998). NF-κB acts during early thymocyte differentiation, T cell lineage commitment, and selection of mature T cells (Gerondakis *et al*, 2014). Notably, NF-κB functions in the neural stem and progenitor cells of the developing and adult brain that are committed to differentiation to promote their proliferation (Zhang *et al*, 2012), in a manner that may resemble NF-κB's role in the hair follicle. While NF-κB has been associated with apoptosis and proliferation in normal tissue development and homeostasis, and NF-κB has been shown to regulate DNA double-strand break repair in response to induced stress (Wu *et al*, 2006; Volcic *et al*, 2012),

our work is the first to our knowledge to suggest a link between these two functions. We suggest that NF-κB may act in the homeostasis of adult hair follicles downstream of *Gata6*, as a protective mechanism employed by these progenitors against naturally-occurring DNA damage associated with rapid proliferation during periods of intense tissue growth.

### *4.3 Future Directions*

As discussed, my work on *Gata6* in the hair follicle suggests that *Gata6* has particular significance in maintaining genomic integrity of highly proliferative cells such as the progenitor cells of the hair follicle matrix. While my work focused on short-term effects of *Gata6* loss, it will be valuable to investigate what happens to the hair follicles and interfollicular epidermis following prolonged depletion of *Gata6*. Due to the health of the mice following *Gata6* loss, I was only able to examine mice up to 10 days post *Gata6* iKO induction. At this point the hair follicles resemble abnormal telogen follicles, with the matrix progenitor cells completely lost due to apoptosis and pigmented cells are found throughout the remaining follicle. Notably, the bulge, which did not display any DNA damage or apoptosis following *Gata6* loss, remained intact at this final stage. Since the early death of *Gata6* iKO mice appears to be due to the effects of *Gata6* loss in other epithelial tissues, it should be possible to examine the long-term effects of *Gata6* loss by applying the tamoxifen topically to the skin. Alternatively, *Gata6* iKO skin may be grafted onto immunocompromised *Nude* mice, however preliminary results from such an experiment has shown inconsistent results due to inflammation following surgery.

Following extensive damage caused by chemotherapy, hair follicles undergo a similar dystrophy as that observed following *Gata6* loss (Hendrix *et al*, 2005). However, upon reaching telogen, these chemotherapeutically damaged follicles promptly initiate anagen to initiate new hair growth. Based on the results of the telogen induced *Gata6* iKO, which fail to initiate anagen, I suspect that following the anagen induction long-term will either result in permanently arrested telogen follicles or further degeneration of the hair follicles. This further degeneration could occur if loss of the progenitor cells causes sustained activation of the bulge stem cells, followed by apoptosis of the resulting progenitor cells.

These long-term *Gata6* loss experiments would also be valuable in examining effects of Gata6 on the interfollicular epidermis. From the results shown in Chapter 3, although GATA6 is expressed in the basal layer of the interfollicular epidermis, DNA damage and apoptosis was never observed. It may be interesting to see investigate whether any changes occur in the epidermis over longer periods of time. Additionally, the interfollicular epidermis has recently been shown to contain distinct fast and slow dividing stem cell populations (Sada *et al*, 2016; Gomez *et al*, 2013). Preliminary analysis of these two populations shows that *Gata6* is preferentially expressed in the fast-dividing lineage. The fast and slow dividing populations can be distinguished based on the markers *Slc1ac* and *Dlx1* (Sada *et al*, 2016). *Gata6*$^{fl/fl}$ mice may be crossed with either *Slc1a3-CreER* or *Dlx1-CreER*, to examine the differential effects of *Gata6* on these two kinetically distinct populations. This would be valuable in understanding if *Gata6* is necessary for proliferation generally or if there is particular significance of *Gata6* upregulation in relation to proliferation rate.

# REFERENCES


Barker N, van Es JH, Kuipers J, Kujala P, Cozijnsen M, Haegebarth A, Korving J, Peters PJ & Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449:** 1003–1007

Behrens A, van Deursen JM, Rudolph KL & Schumacher B (2014) Impact of genomic damage and ageing on stem cell function. *Nat Cell Biol* **16:** 201–207

Bhakar AL, Tannis L-L, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S & Barker PA (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. *J. Neurosci.* **22:** 8466–8475

Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, Brisken C, Minucci S, Di Fiore PP & Pelicci PG (2009) The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* **138:** 1083–1095

Gerondakis S, Fulford TS, Messina NL & Grumont RJ (2014) NF-κB control of T cell development. *Nat. Immunol.* **15:** 15–25

Gomez C, Chua W, Miremadi A, Quist S, Headon DJ & Watt FM (2013) The Interfollicular Epidermis of Adult Mouse Tail Comprises Two Distinct Cell Lineages that Are Differentially Regulated by Wnt, Edaradd, and Lrig1. *Stem Cell Reports* **1:** 19–27

Hendrix S, Handjiski B, Peters EMJ & Paus R (2005) A guide to assessing damage response pathways of the hair follicle: lessons from cyclophosphamide-induced alopecia in mice. *J Invest Dermatol* **125:** 42–51

Hogan BLM, Barkauskas CE, Chapman HA, Epstein JA, Jain R, Hsia CCW, Niklason

L, Calle E, Le A, Randell SH, Rock J, Snitow M, Krummel M, Stripp BR, Vu T, White ES, Whitsett JA & Morrisey EE (2014) Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. *Cell Stem Cell* **15:** 123–138

Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R & Karin M (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* **284:** 316–320

Hua G, Thin TH, Feldman R, Haimovitz-Friedman A, Clevers H, Fuks Z & Kolesnick R (2012) Crypt base columnar stem cells in small intestines of mice are radioresistant. *Gastroenterology* **143:** 1266–1276

Inomata K, Aoto T, Binh NT, Okamoto N, Tanimura S, Wakayama T, Iseki S, Hara E, Masunaga T, Shimizu H & Nishimura EK (2009) Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. *Cell* **137:** 1088–1099

Insinga A, Cicalese A & Pelicci PG (2014) DNA damage response in adult stem cells. *Blood Cells Mol. Dis.* **52:** 147–151

Insinga A, Cicalese A, Faretta M, Gallo B, Albano L, Ronzoni S, Furia L, Viale A & Pelicci PG (2013) DNA damage in stem cells activates p21, inhibits p53, and induces symmetric self-renewing divisions. *Proc Natl Acad Sci USA* **110:** 3931–3936

Janssens S & Tschopp J (2006) Signals from within: the DNA-damage-induced NF-kappaB response. *Cell Death Differ.* **13:** 773–784

Lamm N, Ben-David U, Golan-Lev T, Storchová Z, Benvenisty N & Kerem B (2016)

Genomic Instability in Human Pluripotent Stem Cells Arises from Replicative Stress and Chromosome Condensation Defects. *Cell Stem Cell* **18:** 253–261

Lentjes MHFM, Niessen HEC, Akiyama Y, de Bruïne AP, Melotte V & van Engeland M (2016) The emerging role of GATA transcription factors in development and disease. *Expert Rev Mol Med* **18:** e3

Lezoualc'h F, Sagara Y, Holsboer F & Behl C (1998) High constitutive NF-kappaB activity mediates resistance to oxidative stress in neuronal cells. *J. Neurosci.* **18:** 3224–3232

Li Q, Estepa G, Memet S, Israel A & Verma IM (2000) Complete lack of NF-kappaB activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation. *Genes & Development* **14:** 1729–1733

Li Q, Lu Q, Hwang JY, Büscher D, Lee KF, Izpisua-Belmonte JC & Verma IM (1999a) IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes & Development* **13:** 1322–1328

Li Q, Van Antwerp D, Mercurio F, Lee KF & Verma IM (1999b) Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science* **284:** 321–325

Mizrahi K, Kagan S, Stein J, Yaniv I, Zipori D & Askenasy N (2014) Resistance of hematopoietic progenitors to Fas-mediated apoptosis is actively sustained by NFκB with a characteristic transcriptional signature. *Stem Cells Dev.* **23:** 676–686

Petroni M, Sardina F, Heil C, Sahún-Roncero M, Colicchia V, Veschi V, Albini S, Fruci D, Ricci B, Soriani A, Di Marcotullio L, Screpanti I, Gulino A & Giannini G (2016) The MRN complex is transcriptionally regulated by MYCN during neural cell proliferation to control replication stress. *Cell Death Differ.* **23:** 197–

206

Rudolph D, Yeh WC, Wakeham A, Rudolph B, Nallainathan D, Potter J, Elia AJ & Mak TW (2000) Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes & Development* **14:** 854–862

Sada A, Jacob F, Leung E, Wang S, White BS, Shalloway D & Tumbar T (2016) Defining the cellular lineage hierarchy in the interfollicular epidermis of adult skin. *Nat Cell Biol* **18:** 619–631

Sotiropoulou PA, Candi A, Mascré G, De Clercq S, Youssef KK, Lapouge G, Dahl E, Semeraro C, Denecker G, Marine J-C & Blanpain C (2010) Bcl-2 and accelerated DNA repair mediates resistance of hair follicle bulge stem cells to DNA-damage-induced cell death. *Nat Cell Biol* **12:** 572–582

Sotiropoulou PA, Sotiropoulou PA, Karambelas AE, Debaugnies M, Candi A, Bouwman P, Moers V, Revenco T, Rocha AS, Sekiguchi K, Jonkers J & Blanpain C (2012) BRCA1 deficiency in skin epidermis leads to selective loss of hair follicle stem cells and their progeny. *Genes & Development* **27:** 39–51

Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T, Sanjo H, Yoshikawa K, Terada N & Akira S (1999) Limb and skin abnormalities in mice lacking IKKalpha. *Science* **284:** 313–316

Turco MC, Romano MF, Petrella A, Bisogni R, Tassone P & Venuta S (2004) NF-kappaB/Rel-mediated regulation of apoptosis in hematologic malignancies and normal hematopoietic progenitors. *Leukemia* **18:** 11–17

Volcic M, Karl S, Baumann B, Salles D, Daniel P, Fulda S & Wiesmüller L (2012) NF-κB regulates DNA double-strand break repair in conjunction with BRCA1-

CtIP complexes. *Nucleic Acids Res* **40:** 181–195

Wu Z-H, Shi Y, Tibbetts RS & Miyamoto S (2006) Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. *Science* **311:** 1141–1146

Zhang Y, Goss AM, Cohen ED, Kadzik R, Lepore JJ, Muthukumaraswamy K, Yang J, DeMayo FJ, Whitsett JA, Parmacek MS & Morrisey EE (2008) A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. *Nat Genet* **40:** 862–870

Zhang Y, Liu J, Yao S, Li F, Xin L, Lai M, Bracchi-Ricard V, Xu H, Yen W, Meng W, Liu S, Yang L, Karmally S, Liu J, Zhu H, Gordon J, Khalili K, Srinivasan S, Bethea JR, Mo X, et al (2012) Nuclear factor kappa B signaling initiates early differentiation of neural stem cells. *Stem Cells* **30:** 510–524