Gata6 promotes hair follicle progenitor cell renewal by genome maintenance during proliferation

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Abstract

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 level and NF- κ B 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 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.

Keywords DNA damage; Gata6; hair follicle; progenitor matrix cell; replication stress

Subject Categories Development & Differentiation; DNA Replication, Repair & Recombination; Stem Cells

DOI 10.15252/embj.201694572 | Received 16 April 2016 | Revised 30 September 2016 | Accepted 28 October 2016 | Published online 1 December 2016

The EMBO Journal (2017) 36: 61–78

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 & Tumber, 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 stem cell-intrinsic (i.e. transcription factors) and stem cell-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 cancer and 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 is 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

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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 (Legu & Nicolas, 2005; Legu 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 (Legu & Nicolas, 2005; Legu 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 Fig 1A.

Specifically, *Nfatc1*, *Tcf3/4*, and *Foxc1* are important for maintaining bulge stem cell quiescence (Horsley et al, 2008; Lien et al, 2014; Lay et al, 2016; Wang et al, 2016), while *Tbx1*, *Sox9*, *Lhx2*, and *Gli2* are known to promote bulge cell self-renewal (Chen et al, 2012; Folgueras et al, 2013; Hsu et al, 2014; Kadaja 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 (Osorio et al, 2008, 2011; Hoi et al, 2010; Lee et al, 2013, 2014). *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*,

Figure 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). Arrowhead indicates Gata6 expression in the hair germ.
- 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.
- Data information: Scale bars: 30 μ m.

Msx2, *Hoxc13*, and *Lef1* are known to promote differentiation toward the hair shaft lineage (Zhou *et al*, 1995; DasGupta & Fuchs, 1999; Merrill *et al*, 2001; Jave-Suarez *et al*, 2002; Ma *et al*, 2003; Lowry *et al*, 2005; Weiner *et al*, 2007; Hwang *et al*, 2008; Hu *et al*, 2010a; Kim & Yoon, 2013), while *Cutl1*, *Gata3*, and *Foxn1* promote differentiation toward the IRS lineage (Ellis *et al*, 2001; Kaufman *et al*, 2003; Cai *et al*, 2009).

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 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). During anagen, Sonic hedgehog (*Shh*) and Wnt signaling are implicated in matrix cell proliferation (St-Jacques *et al*, 1998; Wang *et al*, 2000; Oro & Higgins, 2003; Choi *et al*, 2013; Hsu *et al*, 2014).

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 (Morrissey *et al*, 1998; Yang *et al*, 2002; Zhao *et al*, 2005, 2008; Zhang *et al*, 2008; Beuling *et al*, 2011, 2012) 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 (Shureiqi *et al*, 2007; Kwei *et al*, 2008; 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.

Results

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 (Legué & Nicolas, 2005; Zhang *et al*, 2009; 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 (100 μ g/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 (20 μ g/g body) produced single-color-single lineage patterns (Fig 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 EV1A and 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 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 1C and D—top panel). Moreover, expression was high in the differentiated hair layers of inner root sheath (Fig 1C and D). *Gata6* was also present in the infundibulum (the upper portion of the hair follicle) and in the inter-follicular epidermis (Figs 1C and EV1C). In the bulge, *Gata6* was detectable in rare cells in the lower zone bordering the hair germ during early anagen, but was absent from the bulge during stages of HFSCs self-renewal (early and mid-anagen) (Fig 1C). These data suggested that *Gata6* might play a role in matrix cell formation, proliferation, and differentiation.

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^{f/f}* 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 1D) and epidermis (Fig EV1C). Control littermate mice without TM induction (*K14-CreER^{T2}; Gata6^{f/f}* injected with oil), without Cre expression (*Gata6^{f/f}* 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 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 2A). 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 (Figs 2B and EV2A). This was true in all hair follicles from iKO and WT mice

Figure 2. Gata6 is necessary for anagen induction 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/genotype).
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 Fig EV2D.

Data information: Scale bars: 30 μ m.

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, but also 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 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 1D, 2C and EV2B and 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 EV2C). 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 a telogen-like state.

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 whether 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 (Figs 3 and EV3A–D), when Gata6 expression was completely lost from the hair follicle as indicated by our staining (Fig 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 whether 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 3 days after tamoxifen induction, when Gata6 is normally lost in the matrix (Fig 1D) and sacrificed at 12 h and at 3 days after injection by which time Gata6 is also lost from the differentiated lineages (Fig 3A). As expected, after 12 h of chase, the BrdU labeling is predominantly located in the matrix and this was true in both WT and iKO mice (Figs 3B and EV3E). As the matrix cells differentiate, they feed upwards into the IRS and hair shaft. Indeed, after 2-day 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 (Figs 3D and E, and EV3E). 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 3F and 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, 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.

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 γ H2A.X, which localizes to double-strand breaks, and for the apoptosis marker activated caspase-3 (Fig 4A). Gata6 iKO hair follicles displayed frequent matrix cells that were either caspase-3⁺/ γ H2A.X⁺ (indicative of cell death) or caspase-3⁻/ γ H2A.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 4B and C). These data suggested that Gata6 loss results in drastic DNA damage 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 1D). Neither DNA damage nor apoptosis was found within the bulge stem cells (Fig EV4); this was expected since these cells do not express Gata6 during anagen (Fig 1C). Additionally, although the inter-follicular epidermis and infundibulum express Gata6 (Figs 1C and EV1C), neither DNA damage nor apoptosis was observable (Fig EV4), 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 (Legu & Nicolas, 2005), we quantified the distribution of caspase-3 staining in different regions of the matrix (Fig 4D). 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 (Legu & Nicolas, 2005). Careful quantification of the frequency of caspase-3⁺ 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 TM induction (Fig 1D).

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 h before sacrifice (Fig 4E and F). As expected based on the proximity to the DP (Legu & 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 4E and 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.

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 5A and B). While large colonies are seeded by keratinocyte 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,

Figure 3. 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 3 days after TM induction and 12 h after BrdU labeling or 5 days after TM induction and 2 days after BrdU labeling stained with markers as indicated in corresponding color. Scale bars: 30 μ m. Arrowheads indicate colocalization of BrdU with AE13 (D) and AE15 (E).

Figure 4. Hair follicle progenitors show impaired proliferation and increased DNA damage and apoptosis following loss of Gata6.

- A** Hair follicles exhibit immunostaining signal for DNA damage marker γ H2A.X (red) and apoptosis marker caspase-3 (green) as early as 1 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 γ H2A.X-positive cells of the epidermis but is found in some iKO hair follicle matrix cells. Right panels show single-color images as indicated on top. Scale bars: 30 μ m.
- B** Quantification (average $\pm$ SD) of the percentage of apoptotic cells per matrix as determined by counting of caspase-3⁺ cells ($n = 50$ follicles from three mice per group).
- C** Quantification (average $\pm$ SD) of the percentage of DNA damaged cells per matrix as determined by counting of γ H2A.X⁺ caspase-3⁻ cells ($n = 50$ follicles from three mice per group).
- D** Regional distribution of apoptosis was analyzed by dividing the matrix into four compartments as shown in the color legend and counting the fraction of caspase-3⁺ cells for each matrix compartments (right; $n = 50$ follicles from three mice per group).
- E, F** Proliferation in the matrix compartments shown in (D) was assessed by BrdU labeling for 1 h, followed by staining (E), and quantification (F). Box spans the first quartile (Q1, bottom) to the third quartile (Q3, top), the horizontal line within the box represents the median, the whiskers extend from (Q1 or Q3 + 1.5)*(Q3-Q1), and points represent outliers outside of the previously defined range (interior cells labeled with solid bars, exterior cells labeled with striped bars). Scale bars: 30 μ m.

Data information: Unpaired t-test: * P -value < 0.05 . $n = 20$ follicles from 2 mice per group.

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 5D), 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 h following TM addition to the culture medium (Fig 5C). As with the hair follicle matrix cells *in vivo*, iKO cultured keratinocytes rapidly acquired DNA damage indicated by γ H2A.X foci staining, as early as 6 h post-induction (Fig 5E and G). We also confirmed the presence of DNA damage in keratinocytes by comet assays, using WT cells with 1-min high UV dose irradiation as positive controls (Fig 5F and H). Apoptosis was not detectable in iKO cells 6 h post-induction, although it was readily detectable in our control WT cells treated with high UV doses and cultured for 24 h (Fig 5E). These results indicated that DNA damage is an early and rapid response to Gata6 loss. Cell cycle analysis of keratinocytes at 24 h 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 to the observed DNA damage induced by Gata6 loss (Fig 5I and J). These effects on the cell cycle were also detectable in cell culture using a knockdown approach with Gata6-specific shRNA (Appendix Fig S1).

To examine the possible relationship between replication and DNA damage in response to Gata6 iKO, we co-stained EdU-labeled cells with γ H2A.X and found a significantly greater proportion of γ H2A.X⁺ cells associated with EdU⁺ cells than EdU⁻ cells (Fig 5K). Furthermore, to test whether 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 5L). We found DNA damage indicated by γ H2A.X staining was decreased in quiescent cells compared to proliferative cells (Fig 5M). 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.

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 this analysis, we used RNA purified from independent iKO and WT keratinocyte lines (three lines each) 6 h 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 (Fig 6A and Appendix Fig S2). 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 6B and C). Given the 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 6D and 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 (Appendix Table S1). Additionally, we conducted transcription factor binding site enrichment analyses on 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- κ B (Fig 6F and Appendix Table S2). NF- κ B has an established role in regulating proliferation, DNA repair, and apoptosis especially in response to stress (Janssens & Tschopp, 2006). Furthermore, inhibition of the NF- κ B 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- κ B 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 6B and C), which is linked with Eda and NF- κ B signaling and has established roles in hair morphogenesis and in the hair cycle (Mustonen *et al*, 2003; Cui & Schlessinger, 2006; Kloepper *et al*, 2014). We also found the NF- κ B activator TNF- α was down-regulated as well in response to Gata6 loss. TNF- α 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- κ B 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 (Appendix Table S3), a handful, including MCM 3, 6 and 10, were involved in DNA replication and repair and showed expected down-regulation of gene expression by qRT-PCR (Fig 6H). Loss of these genes could potentially explain the observed DNA damage induced by Gata6 iKO in proliferative cells.

Gata6 augments Edaradd expression and NF- κ B signaling *in vivo*

The known interactions among Eda, Edaradd, and NF- κ B (Headon *et al*, 2001) and their implication in DNA damage, proliferation, and

Figure 5. 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 μ m.
- B** Quantification of colony formation from (A), average $\pm$ SD from $n = 3$ mice in each genotype.
- C** Western blot of WT and iKO cultured keratinocytes after 0 or 6 h of tamoxifen induction, blotted with actin and Gata6 antibodies, showing a decrease in Gata6 protein levels after 6 h.
- D** Phase contrast images of WT and Gata6 iKO keratinocytes 3 days after induction with TM ($n > 3$ per genotype). Scale bar: 20 μ m.
- E–H** Images and quantification of DNA damage in cultured keratinocytes based on staining for DNA damage marker γ H2AX (E and G) and comet assay (F and H). Scale bar: 20 μ m. Arrowheads in (F) indicate DNA damaged cell nuclei with comet shape. Quantification of γ H2AX (G) represents average $\pm$ SD from at least 300 cells per condition from three experiments using different cell lines. Unpaired t -test $^*P$ -values: 6 h = 0.01 and 24 h = 0.004. Quantification of comet assay (H) represents average $\pm$ 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 $\pm$ SD ($n = 3$ per genotype). Unpaired t -test $^*P$ -values: G1 = 0.03, S = 5×10^{-4} , G2-M = 7×10^{-4} .
- K** Quantification of the frequency of γ H2AX+ staining in keratinocytes within S-phase (EdU+) or another cell cycle stage (EdU-) following a 20 min EdU pulse labeling (average $\pm$ 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 $\pm$ SD, $n = 4$). (M) Quantification of γ H2AX+ keratinocytes during proliferative and quiescent conditions (average $\pm$ SD, $n = 4$). Unpaired t -test $^*P$ -value = 0.04. Mann–Whitney U -test P -value = 0.06.

Figure 6. Genome-wide transcriptional changes following Gata6 depletion.

- A Heatmap of differentially expressed genes (Wald test 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 $\pm$ SD, *n* = 4). Unpaired *t*-test *P-values: Edaradd = 0.03, Apcdd1 = 0.005, Glis2 = 7×10^{-5} , Ppargc1b = 3×10^{-4} , Rab17 = 2×10^{-5} , Foxn1 = 0.03. Mann-Whitney *U*-test *P*-values: Edaradd = 0.06, Apcdd1 = 0.03, Glis2 = 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 a positive control with elevated ROS. Scale bar: 10 μ m.
- E Quantification of fold change in ROS at 6 and 24 h after TM compared to WT, based on fluorescence intensity of DHE (average $\pm$ 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 down-regulated genes compared to the background rate of occurrences in all gene promoters and Fisher *P*-value of the proportion of down-regulated gene promoters containing each transcription factor binding site compared to the frequency in all gene promoters.
- G Comparison of Gata6 iKO down-regulated genes with genes up-regulated 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 $\pm$ 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.

apoptosis prompted us to further explore a potential upstream role of Gata6 in regulating this pathway in the hair follicle (Fig 7A). 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 6B), Edaradd mRNA was significantly down-regulated in Gata6 iKO skin compared to WT (Fig 7B). Western blotting showed a similar depletion of the Edaradd protein in Gata6 iKO skin compared with WT and reduction of NF- κ B protein phospho-p65 (Serine 536) (Fig 7D). Moreover, immunofluorescence staining and quantification confirmed reduced Edaradd protein and of NF- κ B protein phospho-p65 (Serine 536) within the hair follicle matrix during anagen, in Gata6 iKO skin (Fig 7E–H). Immunofluorescence signal was cytoplasmic as expected from this form of serine phosphorylated NF- κ B (Sakurai *et al*, 2003; Moreno *et al*, 2010).

To test whether 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 (0.1, 1 and 1.5 kb upstream) that contained Gata6 binding sites conserved between mouse and human sequences (Fig 7C). 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- κ B 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 regulation may occur through direct binding of the Edaradd promoter, although additional mechanisms may not be excluded by our data.

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 EV5A). After TM induction of Gata6 iKO, unlike control-transfected cells that did not grow, both Gata6 and Edaradd transfected cultures contained cells survived Gata6 iKO and continued to proliferate forming large colonies (Figs 7I and EV5B). We also examined a potential direct link between Edaradd and the NF- κ B 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- κ B activity appeared consistently down-regulated upon Gata6 loss (Fig 7D–H). This discrepancy may indicate transient and compensatory effects on the activity of NF- κ B *in vitro* due to cells being more dependent on this pathway in the stressful cell culture environment.

Analysis of DNA damage by γ H2A.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 7J). 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 EV5C). 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 consistent with a role in the observed DNA damage rescue (Fig 7K). 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; Alver *et al*, 2014; Becker *et al*, 2014; Miotto *et al*, 2014). Our result 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.

Figure 6.

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

Figure 7. Edaradd–NF-κB signaling is 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 $\pm$ SD, 1 day $n = 3$, 2 days $n = 4$). Unpaired *t*-test *P-values: 1 day = 0.02, 2 days = 0.05. Mann-Whitney *U*-test *P*-values: 1 day = 0.06, 2 days = 0.03.
- C** ChIP-qPCR for Gata6 at three conserved binding sites within the Edaradd promoter. GAPDH served as a negative control. Binding sites 1, 2, and 3 are located 50 bp, 1 kb, and 1.5 kb upstream of the transcription start site, respectively (average $\pm$ 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 in 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. Scale bar: 30 μ m. Unpaired *t*-test *P-value = 3.5×10^{-14} , $n = 100$ follicles from three mice per genotype. Box spans the first quartile (Q1, bottom) to the third quartile (Q3, top), the horizontal line within the box represents the median, the whiskers extend from (Q1 or Q3 + 1.5)*(Q3-Q1).
- G, H** Staining for pNF-κB and quantification show decrease in active NF-κB in the Gata6 iKO matrix. Scale bar: 30 μ m. Unpaired *t*-test *P-value = 2.2×10^{-16} , $n = 50$ follicles from three 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 γ H2AX in cells stably transfected with pMock, pGata6, or pEdaradd plasmids, followed by induction with TM for 6 h (average $\pm$ SD, $n = 4$ with > 500 cells counted per condition). Unpaired *t*-test *P-values = 2×10^{-4} (iKO pGata6 versus pMock), 2×10^{-4} (iKO pEdaradd versus pMock).
- K** qRT-PCR analysis of Mcm10 expression in pMock, pGata6, and pEdaradd WT and iKO expressing cells (average $\pm$ SD, $n = 4$). Unpaired *t*-test *P-values: 8×10^{-4} (iKO pGata6 versus pMock), 0.03 (iKO pEdaradd versus pMock). Mann-Whitney *U*-test *P*-values: 0.03 (iKO pGata6 versus pMock), 0.03 (iKO pEdaradd versus pMock).

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 (e.g. Runx1 and Stat3) have already been implicated in control of early progenitor activation at the telogen/anagen transition (Sano *et al*, 1999, 2000; Osorio *et al*, 2008, 2011; Hoi *et al*, 2010; Lee *et al*, 2014), we focused our attention on the matrix maintenance phenotype during anagen, a process not currently well understood (Fig 1A).

Hair follicle matrix cells have been deemed one of the most proliferative cell populations of any mammalian tissue (Lehrer *et al*, 1998). These cells 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; St-Pierre *et al*, 2006; Weiner *et al*, 2007; Hu *et al*, 2010b; Shimomura *et al*, 2010; Beaumont *et al*, 2011; Giacobbe *et al*, 2013; Shoag *et al*, 2013). 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- κ B and JNK pathways (Headon *et al*, 2001; Kumar *et al*,

2001; Cui *et al*, 2002; Yan *et al*, 2002). Inhibition of NF- κ B 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- κ B, 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, 2010; Omori *et al*, 2008). Additionally, NF- κ B 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- κ B binding sites; of these, 397 also had Gata6 conserved binding sites suggesting possible co-regulation by Gata6 and NF- κ B. Here, we provide evidence that Gata6 is essential for Edaradd expression and for constitutive activation of NF- κ B 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 Edaradd transcription may further control MCM10 expression. Although loss of Gata6 and Edar or NF- κ B both display increased apoptosis, Gata6 iKO hairs undergo a more robust degeneration compared to the premature catagen observed in the latter (Fessing *et al*, 2006; Kloepper *et al*, 2014). This discrepancy is likely due to the down-regulation of other target genes and pathways that may also contribute to Gata6's protective function (Fig 8).

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

Figure 8. Model for the role of Gata6 in hair follicle progenitor cell survival through genome maintenance.

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 (Zhong *et al*, 2011; Lin *et al*, 2012; Shen *et al*, 2013). Our study points toward Edaradd-NF- κ B 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- κ B) on our list of transcription factors potentially associated with Gata6 function (Appendix Table S2). 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.

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 μ g/g body weight; Sigma) or a low dose for inefficient labeling and clonal lineage tracing (20 μ g/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 EV1D). CreER^{T2} was activated by a single intraperitoneal injection of tamoxifen dissolved in corn oil (100 μ g/g body weight). For BrdU experiments, mice were injected intraperitoneally with 50 μ g/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- γ H2A.X (1:200; Millipore 05-636), mouse anti-K14 (1:300; Abcam ab7800), rabbit anti-Edaradd (1:100; Abcam ab124484), and rabbit anti-NF- κ B 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- κ B 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-Ca²⁺ keratinocyte E media (Tumbar, 2006), and were used between passage seven and ten. K14-CreER^{T2} was induced with 1 μ M 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 3 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. Morrissey, 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 Appendix Table S4.

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,b). 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 transgenic skin up-regulated genes was 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 and 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 (Zamboni *et al.*, 2005). Primers used for ChIP-qPCR are listed in Appendix Table S4.

Western blot

Total protein extracts from keratinocytes were subjected to SDS-PAGE and Western blot analysis. Blots were probed with mouse anti-actin (1:8,000; Millipore MAB1501), rabbit anti-GATA6 (1:500; H-92, Santa Cruz sc-9055), rabbit anti-Edaradd (1:1,000; Abcam ab124484), and rabbit anti-NF- κ B p65 Ser536 (1:1,000; 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 ≤ 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 nonparametric 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.

Expanded View for this article is available online.

Acknowledgements

We thank Dr. Pierre Chambon for transgenic mice and Dr. Denis Headon for Edaradd expression vectors. We thank David McDermitt for technical assistance with newborn skin grafting onto Nude mice. Funding for this work was from NIH grants R01AR053201 and R56AR053201 to TT and NIGMS T32GM007273 to ABW.

Author contributions

YVZ conducted microarray identification of Gata6 in the hair follicle, qRT-PCR confirmation, colony formation assay, keratinocyte isolation, and contributed to hematoxylin, Gata6, and γ H2AX staining of skin. All other experiments were conducted by ABW. ABW, YVZ, and TT designed the project. ABW and TT wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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