LHX2 is a direct NF-κB target gene that promotes primary hair follicle placode down-growth

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SUMMARY

The transcription factor nuclear factor-kappa B (NF-κB) plays an essential role in epidermal appendage induction and morphogenesis. In the epidermis of mice lacking NF-κB activity, initiation of primary hair follicle pre-placode formation is observed, but these primitive structures fail to proliferate and generate placodes. NF-κB signaling is known to modulate activity of WNT and SHH signaling at early stages of hair follicle development, but these roles do not fully account for the phenotypes observed when this pathway is blocked. To identify additional NF-κB target genes we developed a novel method to isolate and transcriptionally profile primary hair follicle placodes with active NF-κB signaling. In parallel, we compared gene expression at the same developmental stage in embryos with compromised NF-κB signaling, and wild type littermate controls. In addition to corroborating established NF-κB functions, these analyses uncovered novel NF-κB target genes with potential roles in priming hair follicle placodes for down-growth. Of particular interest, we identify the LIM/homeobox transcription factor LHX2 as a direct NF-κB target gene in this system, and show that loss of LHX2 replicates a subset of the phenotypes seen in embryos with reduced NF-κB signaling. LHX2 and TGFβ2 knockout embryos exhibit very similar abnormalities in hair follicle development, including failure of E-cadherin suppression that is required for follicle down-growth. Consistent with this, we find that TGFβ2 signaling is deficient in embryos with either inhibited NF-κB signaling or loss of LHX2. Furthermore, although TGFβ2 is not a direct LHX2 target gene, we find that exogenous TGFβ2 rescues the hair follicle phenotypes of LHX2 knockout skin explants, indicating that it operates downstream of LHX2. These findings identify a novel NF-κB-LHX2-TGFβ2 signaling axis that is critical for primary hair follicle morphogenesis and may function more broadly in development and disease.

Keywords: NF-κB, LHX2, hair follicle, TGFβ2, cell migration, E-cadherin, EDA-A1, EDAR, mouse, embryo, placode, stem cell

INTRODUCTION

Hair follicle development is initiated by a reciprocal signaling interplay between the surface epithelium and the underlying mesenchyme that results in local epithelial thickenings, the hair follicle placodes (Biggs and Mikkola, 2014; Fuchs, 2007; Hardy, 1992; Schmidt-Ullrich and Paus, 2005; Schneider et al., 2009; Sennett and Rendl, 2012). The regular array of placodes is thought to be mediated by a reaction-diffusion system of competing placode activator and inhibitor morphogens (Bazzi et al., 2007; Jiang et al., 2004; Mou et al., 2006; Sick et al., 2006). Mouse hair follicle development occurs in three major waves, with primary (guard, tylotrich) hair follicles forming at E14.5, and awl/auchene and zigzag hair follicles starting to generate at E16.5 and E18.5 respectively. These distinct waves are differentially regulated at the molecular level. Epidermal and dermal canonical WNT/β-catenin signaling is required to initiate the development of all hair types, while BMP signals generally function to impede placode development (Andl et al., 2002; Botchkarev et al., 1999; Chen et al., 2012; Mou et al., 2006; Oro and Scott, 1998; Zhang et al., 2008; Zhang et al., 2009a). In addition to WNT/β-catenin signaling, primary hair follicle formation specifically depends on the activity of TNF family member EDA-A1 (ectodypslasin-A1) in the epidermis (Headon and Overbeek, 1999; Kere et al., 1996; Laurikkala et al., 2002; Schmidt-Ullrich et al., 2001; Schmidt-Ullrich et al., 2006). *Eda-A1* ligand and its receptor *Edar* (ectodysplasin receptor) are both direct target genes of WNT/β-catenin, and EDA-A1-EDAR interaction results in downstream activation of transcription factor NF-κB in developing primary placodes (Kumar et al., 2001; Laurikkala et al., 2002; Schmidt-Ullrich et al., 2006; Yan et al., 2000; Zhang et al., 2009a).

In the absence of EDA-A1/EDAR/NF-κB signaling, WNT/β-catenin initiates hair follicle placode formation in a messy pre-pattern up to pre-placode stage (hair morphogenesis stage 0/1; (Paus et al., 1999)) in which a subset of placode markers is already expressed, but subsequent down-growth and morphogenesis are arrested (Schmidt-Ullrich et al., 2006; Zhang et al., 2009a). The ill-defined borders of these pre-placodes revealed a role for EDA-A1/EDAR/NF-κB signaling in pattern refinement of early WNT/β-catenin activity in primary hair follicle placodes by up-regulating expression of WNT inhibitors such as *Dkk4* (Bazzi et al., 2007; Fliniaux et al., 2008; Zhang et al., 2009a). Furthermore, a

suggested function for EDA-A1/EDAR/NF-κB in suppressing placode-inhibitory BMP signals within primary placodes may be important to maintain hair follicle fate and prevent premature differentiation (Mou et al., 2006; Pummila et al., 2007). With the exception of recombinant Fc-EDA-A1, TNFα and to some extent high doses of BMP inhibitor Noggin, other potential effectors downstream of EDA-A1 signaling, such as SHH or the chemokines CXCL10 and 11, failed to rescue primary hair follicle placode formation in EDA-A1-deficient embryonic skin explants (Laurikkala et al., 2002; Lefebvre et al., 2012; Pummila et al., 2007; Schmidt-Ullrich et al., 2006). This indicates that the known EDA-A1/EDAR/NF-κB target genes are not sufficient to define the apparently complex role of EDA-A1/EDAR/NF-κB signaling in primary hair placode development. Therefore additional NF-κB-dependent factors must be required to establish proper conditions for placode down-growth beyond initiation, patterning and BMP inhibition.

Previous microarray analyses have identified some NF-κB target genes. However all these studies utilized either whole skin (epidermis and dermis) or skin explants that had been treated with recombinant EDA-A1 or left untreated (Bazzi et al., 2007; Cui et al., 2002; Cui et al., 2006; Laurikkala et al., 2002; Lefebvre et al., 2012; Mou et al., 2006; Pummila et al., 2007). Therefore, placode-specific genes that are expressed at low levels may have been missed. To identify additional genes functioning downstream of NF-κB, we established a gene signature that is both placode keratinocyte-specific, and NF-κB-dependent. To this end we generated a novel NF-κB-EGFP reporter mouse line. This allowed us to isolate and purify hair placode keratinocytes and to set up a detailed gene expression profile specifically of NF-κB-active primary hair placodes. In parallel, to identify NF-κB-dependent genes, we profiled gene expression in NF-κB-inhibited compared with control embryonic mouse epidermis.

Hair follicle placode formation and down-growth is a complex process that depends on changes in local keratinocyte adhesion, migration, polarity and proliferation, as well as modifications in the surrounding ECM. In addition to expected NF-κB targets, such as genes that control NF-κB and WNT signaling, we identified a significant number of new targets including ECM components, genes involved in cell adhesion and migration, and stem cell-associated genes. Of particular interest, we identified the gene encoding the LIM-homeodomain transcription factor LHX2 as a novel NF-κB

target gene in hair follicle placodes. LHX2 was originally shown to control patterning, cell fate decisions and axon guidance during embryonic brain development (Bulchand et al., 2001; Hirota and Mombaerts, 2004; Mangale et al., 2008; Porter et al., 1997; Shetty et al., 2013). However, LHX2 also plays an important role in hair follicle stem cell growth and maintenance within the stem cell niche by regulating cell adhesion and cytoskeletal dynamics (Folgueras et al., 2013; Rhee et al., 2006). A requirement of LHX2 for anagen induction has been indicated as well (Tornqvist et al., 2010). LHX2 is expressed in stage 0 and 1 embryonic hair follicle placodes that also display NF-κB activity, and *Lhx2*-deficient embryos have reduced numbers of hair follicles (Rhee et al., 2006; Tornqvist et al., 2010). These data suggest a role for LHX2 in hair follicle morphogenesis. However, as *Lhx2*-deficient embryos die around E15 - E16, when primary hair follicle development is still ongoing, further detailed morphological and molecular analyses of hair follicle development have not previously been pursued (Porter et al., 1997; Rhee et al., 2006). A more recent study using a mouse line harboring a hypomorphic *Lhx2* allele examined hair follicle development at later time points, but solely confirmed previous assumptions on a possible role of LHX2 in hair follicle development (Tornqvist et al., 2010). Our results now demonstrate an essential role for LHX2 in preparing primary hair pre-placodes for down-growth downstream of EDA-A1/EDAR/NF-κB signaling.

RESULTS

NF-κB-dependent gene signature in primary hair follicle placodes reveals a multifunctional role for NF-κB in hair follicle development

To obtain a gene signature for NF-κB-active primary hair follicle placodes, we generated an NF-κB-responsive reporter mouse line that uses EGFP as read-out for in vivo NF-κB activity (*κ-EGFP*; Fig. 1A; Table S1). The expression pattern of EGFP was identical to that observed in a previously produced NF-κB reporter line (Schmidt-Ullrich et al., 2001; Schmidt-Ullrich et al., 1996), including strong NF-κB activity in developing hair follicle placode keratinocytes (Figs 1A, S1). Epidermis from E14.5 *κ-EGFP* embryos was harvested, EGFP-expressing hair follicle placode keratinocytes were purified by FACS, and total placode keratinocyte RNA was processed for microarray analysis (Figs 1B,C, S1; Table S1). To exclude contamination of our epidermal samples with mesenchymal cell types, we analysed mRNA expression of 4 dermal markers, *Bmp4*, *Col1a1* (collagen 1 alpha1), *Irx1* (Iroquois homebox 1) and *Ngfr* (nerve growth factor receptor; p75NTR) which either revealed absence (*Col1a1*, *Ngfr*), or very low expression levels of these markers (*Bmp4*, *Irx1*; Fig. S2A) (see http://hair-gel.net/ and (Botchkareva et al., 1999; St-Jacques et al., 1998). The resulting NF-κB-active placode-specific gene signature was aligned with an epidermis-specific NF-κB-dependent gene signature that was obtained from microarray transcriptional profiling of epidermis from embryos with suppressed NF-κB activity (*ΔN*) compared with littermate controls at E14.5 (Table S2). These experiments allowed us to identify 74 genes that are specifically expressed in NF-κB-active placode cells, and are directly or indirectly dependent on NF-κB signaling (Fig. 1B).

We validated the data from our profiling experiments by comparison with previously described NF-κB targets and functions in the context of hair follicle formation, confirming the sensitivity and specificity of our experimental strategy (Fig. 1C). These genes included those in the SHH signaling pathway (*Shh*, *Gli1*, *Ptch2*, *Etv4* and *5*); known NF-κB target genes such as NF-κB family member *RelB* (Table S1), *Tnfaip3* (gene of the ubiquitin editing enzyme A20), *Tnf* (TNFα), *Ltb* (Lymphotoxin β), *Foxi3* (Forkhead box I3), chemokine *Cxcl11* and regulators of the WNT pathway, such as *Dkk4* and also a newly identified NF-κB target, *Wif1* (WNT inhibitory factor 1) (Figs 1C, S3A,B,C) (Bazzi et al., 2007;

Cui et al., 2006; Fliniaux et al., 2008; Lefebvre et al., 2012; Lettice et al., 2012; Mao et al., 2009; Schmidt-Ullrich et al., 2006; Shirokova et al., 2013; Zhang et al., 2009a; Zhang et al., 2009b). A striking number of genes that we identified as potential NF-κB target genes encode extracellular matrix (ECM) components (*Frem1* (FRAS1 related extracellular matrix 1), *Mmp9* (Metalloproteinase 9), *Tnc* (Tenascin C)), receptors and genes implicated in cell migration (*Prokr2* (Prokineticin receptor 2), *Nrp2* (Neuropilin 2), *Cd74* (MIF, macrophage migration inhibitory factor)) or are involved in cell-adhesion, such as *Ncam1* and *Madcam-1* (Fig. 1C). While *Frem1*, *Madcam-1* and *Mmp9* have previously been described as NF-κB target genes (Takeuchi and Baichwal, 1995; Yoon et al., 2012; Yoshizaki et al., 1998), *Prokr2*, *Nrp2*, *Cd74* and *Ncam1* are new potential targets. Importantly, novel NF-κB-regulated genes with special relevance for hair biology such as transcription factors *Lhx2* (LIM homeobox protein 2) (Folgueras et al., 2013; Mardaryev et al., 2011; Rhee et al., 2006; Tornqvist et al., 2010), *Sox9* (SRY-box 9), (Nowak et al., 2008; Vidal et al., 2005), *Trps1* (trichorhinophalangeal syndrome 1) (Fantauzzo et al., 2008a; Fantauzzo et al., 2008b; Kunath et al., 2002; Malik et al., 2002; Momeni et al., 2000) and *Sox21* (SRY-box 21) (Kiso et al., 2009) were also identified (Fig. 1C). A role for LHX2 in primary hair follicle placode down-growth will be described in more detail below.

A number of previously suggested or confirmed NF-κB target genes were up-regulated in hair follicle placodes at E14.5, but were not significantly controlled by NF-κB at this time point (Fig. 1C). These comprised BMP signaling regulator *Ctgf* (connective tissue growth factor) (Pummila et al., 2007), *Wnt10b* (Zhang et al., 2009a) and bona fide NF-κB target *Nfkbia* (NF-κB inhibitor of κBα, IκBα) (Le Bail et al., 1993). *Ctgf* expression may also be regulated by WNT/β-Catenin which is very active in hair follicle placodes at E14.5. For *Wnt10b* we showed previously that it is only regulated by NF-κB at E15.5 when primary hair follicle stage 1 placodes enter the germ stage (stage 2) (Zhang et al., 2009a). *Nfkbia* was expected to be down-regulated in *ΔN* embryos, but the lack of differential regulation may be due to detection of the truncated human IκBα (ΔN) by the mouse array used for our analysis (Schmidt-Ullrich et al., 2001; Schmidt-Ullrich et al., 2006).

To verify placode-specific expression and dependence on NF-κB activity, candidate targets were examined by quantitative Real Time-PCR (qRT-PCR) for enrichment in developing primary hair

follicle placodes and for differential expression in *ΔN* versus control epidermis at E14.5 (Figs 1D,E, S3). qRT-PCR revealed a significant dependence on NF-κB activity for all candidate target genes, including those which regulate cell migration and adhesion (Figs 1D,E, 3A, S3A,B). To identify potential direct targets, the genomic regions of differentially expressed genes were screened for putative NF-κB binding sites using the JASPER database (http://jaspar.cgb.ki.se). We also used the ECR browser (http://ecrbrowser.dcode.org) to check for NF-κB binding site conservation across species (Table S3). These analyses verified that 96% of the potential target genes contained at least one conserved NF-κB binding site (Table S3). Potential binding sites were further confirmed by chromatin immunoprecipitation (ChIP) for *Lhx2*, *Sox9*, *Trps1* and *Mmp9* (see below and data not shown; see also Table S3 and Suppl. Material). Interestingly, 57% of the NF-κB-regulated genes found in our gene chip analysis were also identified by ChIP as direct NF-κB targets in Hodgkin lymphoma cell lines (unpublished data; Table S3). This suggests that physiological NF-κB functions required for primary hair follicle placode formation in mice may in part contribute to human tumor growth and/or survival.

Hair follicle placode-specific mRNA expression of identified potential NF-κB target genes was further validated by in situ hybridization (ISH) on control skin and *ΔN* skin samples at E14.5 (Figs 1E, S3C). Interestingly, mRNA expression of *Bmp2* and *Scube1* (signal peptide-CUB domain-EGF-related 1), a potential SHH and BMP signaling regulator (Johnson et al., 2012; Tsao et al., 2013), was expanded in the absence of epidermal NF-κB activity. ISH revealed that expression of *Bmp2* and *Scube1* was ubiquitously expressed in the epidermis of *ΔN* embryos at E14.5, while it was restricted to primary hair follicle placodes in controls (Fig. S3C). This suggests that NF-κB activity may be indirectly required for restricting *Bmp2* and *Scube1* expression to hair follicle placodes. Some of the NF-κB-regulated genes were also expressed in the dermal condensate (*Nrp2*, *Mmp9*, *Tnc*) and at the interface between placode and dermal condensate (*Mmp9*, *Tnc*) (Figs 1E, S3C). This latter group of differentially regulated genes strongly points to a role for NF-κB in ECM modulation and in cell migration in order to allow placode down-growth, and further suggests additional cell non-autonomous mechanisms by which epithelial NF-κB signaling modulates gene expression in other cell types. Note

that 6 genes were specifically up-regulated in the interfollicular epidermis (IFE), but down-regulated by NF-κB in placodes (Table S4). However none of these genes have any known functions in IFE development or maintenance, suggesting that NF-κB is not required for down-regulating important IFE-regulatory genes in order to promote HF formation.

NF-κB directly regulates *Lhx2* expression and acts in concert with LHX2 to control genes involved in cell migration during placode down-growth

While the novel NF-κB targets *Lhx2* and *Sox9* may suggest an interesting role for NF-κB in stem cell biology, these hair follicle stem cell markers have not previously been associated with early stages of placode formation. mRNA expression of *Lhx2* and *Sox9* was strictly dependent on NF-κB activity in primary hair follicle placodes at E14.5 (Figs 2A, S3A,B,C). In line with this, the promoter of each of these genes contains a single NF-κB binding site that was verified by chromatin immunoprecipitation (ChIP; Fig. 2B and data not shown). Although we observed *Sox9* mRNA expression in suprabasal cells of primary hair follicle placodes at E14.5 (Fig. S3C), SOX9 protein expression is not detected prior to E15.5 and does not play a role in hair follicle induction and early morphogenesis, instead being required for formation and maintenance of early and adult bulge stem cells (Kadaja et al., 2014; Nowak et al., 2008; Vidal et al., 2005). We therefore focused on LHX2, as LHX2 protein was readily detected in control hair follicle placodes, was congruent with NF-κB activity, and was absent in the epithelium of *ΔN* embryos at E14.5 (Fig. 2C).

To examine the precise role of LHX2 in hair follicle morphogenesis, we examined primary guard hair placode formation in *Lhx2* KO (*Lhx2* knock-out) embryos at E14.5 and E15.5 compared with control littermates. A previous analysis of *Lhx2*-KO embryos revealed a 40% reduction of developing placodes at E16.5, when secondary hair follicle induction sets in (Rhee et al., 2006). Similarly to *ΔN* embryos, *Lhx2*-KO embryos initiated primary hair follicle pre-placode generation at E14.5 (Fig. 2D) (Schmidt-Ullrich et al., 2006; Zhang et al., 2009a). However, subsequent placode formation went on to stage 1 in which most placodes seemed to be arrested (Fig. 2D). Direct LHX2 target genes that are involved in cell adhesion and cytoskeletal dynamics in the hair follicle stem cell niche suggest a

possible role for LHX2 in directed cell growth (Folgueras et al., 2013). In line with this, quantitative RT-PCR showed that mRNA expression of genes involved in cell migration, such as the previously identified LHX2 target gene *Nrp1* (Folgueras et al., 2013) as well as the novel NF-κB-regulated genes *Nrp2* and *Prokr2*, were markedly down-regulated in the epidermis of *Lhx2*-KO embryos at E14.5 (Fig. 2E). This was further supported by lack of in vivo mRNA expression of *Nrp2* and *Prokr2* in placodes of *Lhx2*-KO embryos when compared to controls at E14.5 (Fig. 2F). Again, a possible contamination of dermal cells in purified epidermal cell samples of *Lhx2-KOs* and controls was ruled out by analyzing mRNA expression of *Bmp4*, *Col1a1*, *Irx1* and *Ngfr* (see Fig. S2B). However, expression of *Nrp2* mRNA was conserved in the dermal condensate, indicating that *Nrp2* expression is differentially regulated in hair follicle placodes and dermal condensates (Fig. 2C,F). Notably, both *Nrp2* and *Prokr2* genes have potential NF-κB and LHX2 binding sites in their promoter regions (Table S3), suggesting that these genes may be regulated synergistically by NF-κB and downstream LHX2.

Lhx2-KO and *ΔN* mice display cell migratory and proliferative defects in down-growing primary hair follicle placodes

To further explore whether NF-κB and LHX2 cooperate in regulating directed placode keratinocyte migration and proliferation, we examined staining for F-actin (filamentous actin) and expression of phospho-FAK (activated focal adhesion kinase) and Ki67 on skin sections at E14.5 (Fig. 3). FAK (also PTK2), a non-receptor tyrosin kinase, regulates focal cell adhesion and directed migration, as well as polarity and proliferation (Frame et al., 2010; Schaller, 2010). FAK is stimulated by growth factors, such as PDGF and EGF, and by integrin/ECM interactions, which lead to activation of FAK by phosphorylation (Sieg et al., 2000). FAK is essential for embryonic development and also plays an important role in epithelial oncogenesis (reviewed in (Sulzmaier et al., 2014)). Moreover, mice deficient in epidermal FAK lack proper hair follicle down-growth and display hair cycle defects (Essayem et al., 2006; Schober et al., 2007). F-actin staining is also typically enhanced during directed cell migration. As expected, primary hair follicle placodes of controls displayed phospho-FAK (pFAK) expression and F-actin staining in the proximal placode border adjacent to the dermal condensate at E14.5 (Fig. 3). However, F-actin and phospho-FAK were absent in the epithelium of

Lhx2-KOs and *ΔN* embryos (Fig. 3). Proliferation marker Ki67 was also expressed in hair follicle placodes of controls but was not detected in *Lhx2*-KO or *ΔN* embryos at E14.5 (Fig. 3). These data strongly suggest that NF-κB and downstream LHX2 are required to generate the appropriate environment for primary hair follicle placode keratinocytes to proliferate and migrate into the underlying dermis.

***Lhx2-KO* and *ΔN* mice show impaired TGFβ signaling and lack of E-cadherin down-regulation**

In order to allow placode down-growth, cells at the very proximal border of the hair follicle placode have to undergo a number of changes, including loss of cell adhesion. This involves local down-regulation of the epithelial cadherin E-cadherin, the transmembrane core of adherens junctions (AJ) (Jamora et al., 2003; Jamora et al., 2005). The TGF-β family of signaling molecules controls cell-cell interactions, cell migration and proliferation. In particular, TGF-β2 promotes hair follicle morphogenesis by inducing expression of the transcriptional repressor Snail and MAPK activity, resulting in local E-cadherin down-regulation (Jamora et al., 2005). Mice lacking TGF-β2 activity display a delay in hair follicle development and a 50% reduction in the numbers of follicles that form (Foitzik et al., 1999; Jamora et al., 2005). These studies only examined secondary hair follicle development which is independent of EDA-A1/EDAR/NF-κB signaling (Schmidt-Ullrich et al., 2001; Schmidt-Ullrich et al., 2006). During primary hair follicle placode growth, E-cadherin down-regulation must be independent of Snail because we did not detect any *Snail* (*Snai1*) expression in our gene profiling analysis (Table S1), and a previous publication only revealed Snail protein expression at E16.5 (Jamora et al., 2005). By contrast *Snai3* was upregulated (1.6x) in primary hair placodes when compared with interfollicular epidermis at E14.5 (Table S1), suggesting that it might substitute for Snail in primary hair placode growth.

Interestingly, *Tgfb2*-KO mice have a hair follicle developmental phenotype that is very similar to that of *Lhx2*-KO mice (data not shown and Figs 3, 4) (Foitzik et al., 1999). This prompted us to ask whether TGF-β signaling is affected in *Lhx2*-KO and *ΔN* embryonic skin at E14.5. In control embryos, TGF-β2 protein expression was detected in the suprabasal layer of the epidermis, in the entire placode

border adjacent to the dermis and in the dermal condensate, the future dermal papilla (Fig. 4A, upper panels) (Jamora et al., 2005). Phospho-SMAD2 (pSMAD2) expression, which provides a sensitive read-out parameter for active TGF-β signaling, was observed in the entire epidermis including hair follicle placodes, and also in the dermal condensate of controls (Fig. 4A, upper panels). In *Lhx2*-KO embryos TGF-β2 protein was still expressed in the suprabasal epidermis, but pSMAD2 expression was reduced at this site when compared to controls (Fig. 4A, upper panels). In hair follicle placodes and dermal condensates of *Lhx2*-KO embryos pSMAD2 expression was strongly reduced and TGF-β2 protein expression was undetectable in both compartments at E14.5. Similarly to *Lhx2*-KO's, *ΔN* embryos only expressed TGF-β2 protein throughout the epidermis, but pSMAD2 expression was also strongly decreased (Fig. 4A, upper panels). However qRT-PCR indicated that *Tgfb2* is not a direct target gene of either NF-κB or LHX2 in epidermis or dermis (Figs 1C, 4C,D, Table S2). Furthermore, the *Tgfb2* gene lacks binding sites for NF-κB and LHX2 (Table S3). This suggests an indirect control of TGF-β2 protein expression and/or activity by NF-κB and LHX2 in placodes and dermal condensate.

In line with our above findings, E-cadherin expression was absent in proximal placode borders of controls at E14.5, but was readily detectable in placodes of *Lhx2*-KO embryos (Fig. 4A, lower panels). As expected, in *ΔN* embryos E-cadherin expression was observed in the entire epidermis without local down-regulation because primary hair follicle placode formation is barely initiated and only reaches a rudimentary stage 0/1 (Fig. 4A, lower panels) (Schmidt-Ullrich et al., 2006; Zhang et al., 2009a). We also analysed Fibronectin 1 (Fn1) expression, which plays an important role in cell adhesion, migration and proliferation during embryonic development (Schwarzbauer and DeSimone, 2011). *Fn1* mRNA expression was up-regulated in control placodes at E14.5 (Fig. 4B). In contrast, *Lhx2*-KO embryos revealed diminished *Fn1* mRNA expression and in *ΔN* embryos *Fn1* was not detected (Fig. 4B; see also Fig. 1C). Together, these findings suggest that delayed placode formation in *Lhx2*-KO mice and absent placode down-growth in *ΔN* mice are caused in part by decreased TGF-β2 signaling, failure of E-cadherin downregulation, and decreased Fn1 expression.

Recombinant TGF-β2 restores primary HF development in *Lhx2-KO* embryonic skin explants

As TGF-β2 signaling appears to function downstream of LHX2 in primary hair follicle placode formation, we asked whether primary placode growth in *Lhx2*-KO mice could be rescued by treatment of cultured E14.5 skin explants with recombinant TGF-β2. TGF-β2 treatment of E14.5 skin explants from control mice slightly accelerated primary hair follicle placode growth and expression of cell migration and proliferation markers or E-cadherin down-regulation (Fig. S4A,B), consistent with previously published data (Foitzik et al., 1999). TGF-β2-treated *Lhx2*-KO explants showed significantly increased formation of placodes at hair morphogenesis stages 1 and 2 after 24 hours when compared to untreated explants (Fig. 5A). TGF-β2 treatment of *Lhx2*-KO explants not only rescued and enhanced placode down-growth, but also restored TGF-β signaling, down-regulation of E-cadherin and expression of cell migration and proliferation markers pFAK and Ki67 in hair follicle placodes (Fig. 5B). Thus, TGF-β2 activation acts downstream of LHX2 to promote transient E-cadherin down-regulation. By contrast, treatment of *ΔN* skin explant cultures with recombinant TGF-β2 did not rescue hair follicle development (Fig. S4A). This indicates that additional NF-κB targets, likely including growth regulators such as SHH and/or physiological processes such as ECM remodeling (see above) are required downstream of NF-κB activity and cannot be compensated by addition of TGF-β2 alone (Mill et al., 2003; Schmidt-Ullrich et al., 2006; St-Jacques et al., 1998; Zhang et al., 2009a).

DISCUSSION

Here we identified a novel NF-κB-LHX2-TGF-β2 signaling axis which results in E-cadherin down-regulation in primary hair follicles at early stages of their formation, an essential requirement for placode down-growth (Fig. 6; (Jamora et al., 2003; Jamora et al., 2005; Zhang et al., 2009a)). Several EDA-A-dependent target genes have previously been identified and shown to function in hair follicle development. These targets support a function for EDA-A1/EDAR/NF-κB signaling in placode patterning, WNT regulation and BMP suppression, but do not elucidate its role in intrinsic down-growth mechanisms (Bazzi et al., 2007; Cui et al., 2002; Cui et al., 2006; Fliniaux et al., 2008; Lefebvre et al., 2012; Mou et al., 2006; Pummila et al., 2007; Zhang et al., 2009a). Another downstream NF-κB target, SHH, promotes placode growth by up-regulating cyclin-D1 expression (see Fig. 1C) (Mill et al., 2003; Pummila et al., 2007; Schmidt-Ullrich et al., 2006). However this occurs well after induction of EDA-A1/EDAR/NF-κB signaling, consistent with the later arrest of follicle development in *Shh*-deficient compared with NF-κB-inhibited mice (Chiang et al., 1999; Schmidt-Ullrich et al., 2006; St-Jacques et al., 1998). In the current study we therefore sought to identify NF-κB-dependent factors that are specifically required to establish the appropriate conditions for placode down-growth beyond initiation. Our data confirm an essential role for EDA-A1/EDAR/NF-κB signaling in preparing primary hair pre-placodes for down-growth and identify several novel NF-κB target genes. Importantly, we identified LIM homeobox transcription factor LHX2 as a critical new NF-κB-controlled gene which contributes to providing the proper conditions for placode down-growth primarily via activation of TGF-β2 signaling, a known hair placode growth inducer (summarized in Fig. 6) (Foitzik et al., 1999; Jamora et al., 2005).

We further show that NF-κB regulates genes involved in ECM remodeling (*Frem1*, *Mmp9*, *Tnc*), cell migration (*Nrp2*, *Prokr2*, *Cd74*) and adhesion (*Ncam1*, *Madcam1*) in primary hair placodes at E14.5. We have previously observed loss of structural organization at sites of placode induction when NF-κB activity is suppressed (Schmidt-Ullrich et al., 2006). This may be due in part to reorganization of the epidermis (Schmidt-Ullrich et al., 2006). The characteristic structural organization of epidermal keratinocytes and of the underlying dermal condensate at sites of placode formation is most likely an

important prerequisite for subsequent down-growth. It has also recently been suggested that EDA-A1/EDAR/NF-κB signaling is involved in modulating cell motility and placodal fate decisions resulting in early placode formation prior to down-growth, as mice with forced epidermal EDA-A1expression displayed increased cell motility in the interfollicular epidermis and in future areas of hair follicle formation (Ahtiainen et al., 2014). However excess EDA-A1 expression generally results in premature and aberrant placode formation and may thus not reflect the physiological role of endogenous EDA-A1 signaling in hair placode induction (Ahtiainen et al., 2014; Mustonen et al., 2003). Our detailed NF-κB-dependent gene signature in hair placodes supports a mandatory role for NF-κB signaling in ECM remodeling and cell migration and is consistent with our previous findings that NF-κB is required for placode pattern refinement and down-growth rather than rudimentary pre-placode formation and hair fate decisions, which are both dependent on canonical WNT signaling (Andl et al., 2002; Schmidt-Ullrich et al., 2006; Zhang et al., 2009a). Overall, our study illuminates a role for NF-κB in primary hair follicle development that extends well beyond the previously described functions in terms of molecular controls in tissue remodeling, and may be relevant for understanding other epithelial-mesenchymal tissue interaction systems such as those that occur in tumor growth.

After completion of our studies an RNA-seq-based transcriptome of hair follicle progenitors was published (Sennett et al., 2015) (see also http://hair-gel.net/). This useful resource confirms our findings regarding placode-specific expression of *Lhx2*, *Dkk4*, *Foxi3*, *Shh*, *Tnfaip*, *Wnt10b*, *Fgf20* or *Ascl4*. However our in situ hybridization and qPCR studies revealed several inconsistencies, for instance regarding the location of *Fn1*, *Prokr2*, *Sox9*, *Nrp2*, *Frem1*, *Ncam1*, and *Trps1* expression. Sennett et al.'s RNA-seq data indicate exclusive expression of these genes in the dermal compartment; however our in situ hybridization and qPCR studies reveal that *Sox9*, *Prokr* and *Fn1* expression is confined to the hair placode, while *Nrp2*, *Frem1*, *Ncam1* and *Trps1* are expressed in both placode and dermal condensate (Figs 1D,E, 2E,F, 4B, S2A,B,C). These discrepancies highlight the need to verify gene expression patterns inferred from FACS analyses and transcriptional profiling using independent approaches.

Our data suggest that EDA-A1/EDAR/NF-κB controls primary placode down-growth at various levels, including ECM remodeling, and downstream expression of LHX2 that leads to activation of TGF-β2 signaling and subsequent E-cadherin down-regulation (Fig. 6). In terms of hair placode growth delay, *Lhx2-KO* mice strongly resemble mice deficient in *Tgfb2* expression (Fig. 2) (Foitzik et al., 1999), and similarly to *Tgfb2*-KOs (Jamora et al., 2005), E-cadherin down-regulation was absent in stage 1 placodes of *Lhx2-KO* embryos at E14.5. The importance of E-cadherin down-regulation for hair placode down-growth, which is dependent on TGF-β2 signaling, was demonstrated previously using mice with forced epidermal E-cadherin expression in which placode growth was totally blocked (Jamora et al., 2003; Jamora et al., 2005). Although neither NF-κB nor LHX2 appear to control expression of the *Tgfb2* gene or components of the pathway (Figs 1, 4C,D, Table S1) (Folgueras et al., 2013), activation of TGFβ2 signaling is downstream of both transcription factors and directly or indirectly depends on the transcriptional activity of LHX2. As almost all cell types express TGFβ receptors, TGFβ activation is tightly controlled. The TGFβ protein is produced as a latent inactive form that is mainly activated by binding to integrins (Worthington et al., 2011). It is thus conceivable that NF-κB and particularly LHX2 are indirectly responsible for the release of TGF-β2 from its latent inactive form. Initiation of placode down-growth leads to changes in the ECM at the proximal placode border. These changes are likely to be controlled by NF-κB and downstream LHX2 (see above) and may make local integrins available for binding to latent TGFβ complexes. Another reason for lack of TGF-β2 activity in *ΔN* and *Lhx2-KO* mice may be loss of Nrp2 (Neuropilin 2) expression in hair placodes of both mouse models. It was recently shown that Neuropilins can activate the latent TGFβ complex (Glinka et al., 2011). Furthermore, Neuropilins can act as co-receptors for TGFβ receptors and increase the response to latent and active TGF-β (Glinka et al., 2011). In *Lhx2-KO* mice *Nrp2* mRNA expression was absent in the epidermis, however it was still expressed in the dermal condensate, which may be sufficient to induce TGF-β signaling and placode down-growth when *Lhx2-KO* skin explants are treated with recombinant TGF-β2. Further investigation of the mechanisms by which NF-κB functions to mediate local changes in cell adhesion and ECM modulation in placode down-growth will be highly interesting in light of the important roles of NF-κB in tumor growth.

In mature follicles, LHX2 controls hair follicle stem cell maintenance and proliferation by regulating cytoskeletal organization, polarity and cell adhesion within the niche (Folgueras et al., 2013; Mardaryev et al., 2011; Rhee et al., 2006). Placode growth also involves changes in cell adhesion, polarity and proliferation and we show that *Lhx2-KO* mice have delayed primary hair placode growth. Thus, in addition to its role in promoting TGFβ2 signaling, LHX2 may have some analogous functions in hair follicle stem cell maintenance and in early primary placode down-growth. Exploring the molecular connections between embryonic hair follicle precursors and adult stem cells will be a fascinating area for future studies.

MATERIALS AND METHODS

Generation of transgenic mice and animal experiments

All aspects of animal care and experimental protocols were approved by the Berlin Animal Review Board (Reg. G 0261/02, G 0077/08, G 0082/13 and X 9013/11). The EGFP cDNA was cloned immediately downstream of an artificial NF-κB responsive promoter, which has been described previously (Schmidt-Ullrich et al., 1996). The construct was linearized, purified and used for pronuclear microinjection to generate *B6-Tg(κ-EGFP)3Pt/Rsu* mice (here referred to as *κ-EGFP*). Preexisting mouse strains used for this study have been described earlier: *129;129P2-ctnnb1*$^{tm(NFKBIA\Delta N)1Rsu}$ (Δ*N*) (Schmidt-Ullrich et al., 2001), *B6-Lhx2*^{tm1Hwe} (*Lhx2* knock-out mice, here referred to as *Lhx2*-KO, were kindly provided by H. Westphal) (Porter et al., 1997) and *B6CBACa-A*$^{w-J}$*/A-Ta* (*tabby*, *ta/ta*) (*Eda-A1*-mutant mice, kindly provided by I. Thesleff) (Falconer, 1952). Mice and embryos were genotyped by PCR of genomic DNA. Heterozygous *Lhx2* knock-out mice were crossbred to obtain homozygous mutants, which were collected before embryonic day (E16.5).

Isolation of primary placode keratinocytes by flow cytometry

Back skin from E14.5 *κ-EGFP* embryos was dissected and treated overnight with dispase (BD Bioscience, 2.5 Units/mL) at 4°C, which selectively separated the epidermis with primary placodes from the underlying dermis. The epidermal fraction was treated with 10mM EDTA, and cell suspensions were subsequently strained (35μM pores; BD Bioscience). Further purification of placode keratinocytes was performed using a FACSAria III system, equipped with FACS DiVa software (BD Biosciences). Cells were gated for single events and viability, and then sorted by EGFP expression. Purity of the sorted placode keratinocyte population was determined by post-sort FACS analysis which typically exceeded 95% (Fig. S1). Back skin from E14.5 *κ-EGFP* embryos of various different litters was prepared this way and pooled for 5 independent microarray experiments.

Microarray and qRT-PCR

Total RNA from either epidermal keratinocytes of 5 control or 5 Δ*N* embryos at E14.5, or of FACS-purified EGFP-positive (placode) or EGFP-negative (IFE) epidermal keratinocytes from *κ-EGFP* embryos at E14.5 (see above) was isolated using the Absolutely RNA Microprep Kit (Agilent Technologies) and then processed with the WT Expression Kit (Ambion) and the WT Terminal Labeling and Hybridization Kit (Affymetrix). Processed RNA was hybridized to the Mouse Gene 1.0 ST Array (Affymetrix). Five biological replicates for each sample were statistically analyzed with the multi-factor ANOVA test using Partek Genomic Suites software (Partek). The complete microarray data are listed in supplementary Table S1. For quantitative real-time PCR (qRT-PCR), total RNA was used to generate cDNA by means of the iScript cDNA Synthese Kit (Bio-Rad). qRT-PCR primers were designed using Primer3 software (see supplementary Table S2). Reactions were performed in triplicates using the GoTaq qPCR Master Mix (Promega) and a CFX 96 Realtime PCR Detection system (Bio-Rad). Differences between samples were calculated using the CFX Manager software (Bio-Rad) based on the ΔΔCt equitation method, and were normalized to three house-keeping genes (*Actb*, *Gapdh* & *Hmbs*). Statistical significance was estimated using unpaired Student's t-test.

Histology, immunofluorescence and *in situ* hybridization

Back skin samples were fixed in 4% paraformaldehyde/MEM or in Bouin's fixative overnight at 4°C, and were either directly embedded in Tissue Tek O.C.T. or dehydrated and paraffin-embedded. Routine H&E staining was done for morphological evaluation. The progress of hair follicle development was assessed by morphometry using the classification of hair follicle stages by (Hardy, 1992) and (Paus et al., 1999). Cryosections were used for cytoskeleton staining with Phalloidin coupled to AlexaFluor488 (Life Technologies, dilution 1:100). Immunofluorescence and *in situ* hybridization on paraffin sections were performed as described previously (Zhang et al., 2009a). Antibodies and dilutions used: P-Cadherin, rat, Invitrogen (13-2000Z) 1:400; EGFP, chicken, Abcam (ab13970) 1:1600; LHX2, goat, Santa Cruz (Sc-19344) 1:400; KRT14 rabbit & chicken, Convance (AF64 & CK14) 1:2000; EDAR, goat, R&D Systems (AF745) 1:200; p-SMAD2, rabbit, Cell

Signaling (#3101) 1:400; phospho-FAK (Y397), rabbit, Santa Cruz (sc-11765R) 1:400; Ki67, rabbit, Abcam (ab15580) 1:400; E-Cadherin, mouse, BD Bioscience (#610181) 1:100; TGF-β2, mouse, Abcam (ab36495) 1:100; Digoxigenin-AP Fab fragments, sheep, Roche (#11093274910) 1:1000.

Probe sequences used for *in situ* hybridization are provided in the Supplementary Material. Images were obtained by a conventional or confocal Zeiss microscope.

Chromatin Immunoprecipitation (ChIP)

1 x 10^6 EGFP-positive (placode) or EGFP-negative (IFE) epidermal keratinocytes from E14.5 *κ-EGFP* embryos were fixed in 1% formaldehyde for 10 min at room temperature. Subsequent cell lysis, sonification and ChIP assays were performed using the MAGnify Chromatin Immunoprecipitation System (Invitrogen). For each immunoprecipitation, cells were incubated with anti-p65 antibody (Santa Cruz, rabbit, SC-372-X). qRT–PCR was then performed to visualize specific enrichment of potential NF-κB binding regions**.** Ct values of the region of interest and a control region (TSS of *Gapdh*) were measured in the input and ChIP sample, and ΔCt values were calculated. Three replicates were measured and mean±s.e.m. were calculated. Statistical analysis was performed using unpaired Student's t-test. Primers are listed in Supplementary Material.

Embryonic skin culture

Back skin explants from *Lhx2*-KO and control littermates were collected at E14.5, transferred onto 0.1 µm PVDF membranes (Millipore) and cultured using Advanced DMEM:F12 (Gibco) supplemented with 0.5 mM L-glutamine and 100 units/ml penicillin/streptomycin. Skins were kept in a floating culture. Explants were either treated with 100 ng/ml human recombinant TGFβ2 (PeproTech) or left untreated. After 24 hours, explants were harvested and prepared for paraffin embedding (see above). Progression of hair follicle development was monitored in three independent biological replicates. For each replicate, the number of placode and germ stage follicles per microscopic field (100x) was calculated in 30 H&E-stained sections. p-values were calculated using unpaired Student's t-test.

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Competing interests

None.

Author contributions

P.T. designed and performed the experiments, and analysed the data. R.P. designed experiments, analysed the data and edited the manuscript. S.E.M. analysed the data and edited the manuscript. C.S. analysed the data and edited the manuscript. R.S.-U. oversaw the entire project, designed experiments, analysed the data, and wrote the paper.

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Figures

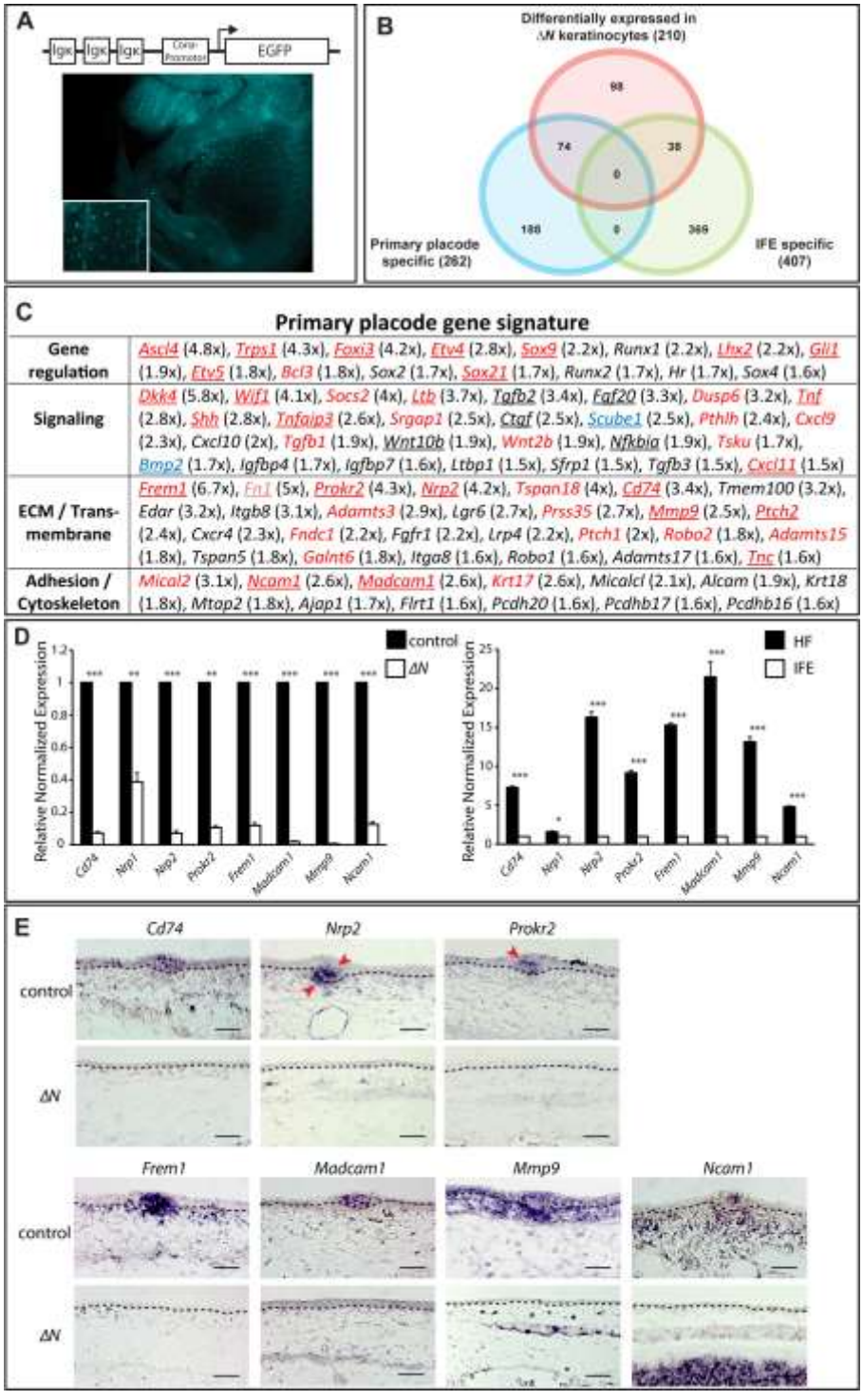

Fig. 1 NF-κB-dependent gene signature in primary hair follicle placodes reveals a multifunctional role. (A) Generation of an NF-κB-responsive EGFP reporter mouse line (*κ-EGFP*).

EGFP expression was observed in the developing HF and in blood vessels of the skin at E14.5. **(B)** Venn diagram illustrates overlap of genes up- or down-regulated in primary hair follicle placodes with differentially expressed genes in Δ*N* versus control epidermal keratinocytes. For full list of microarray data see supplementary material table S1. **(C)** Primary hair follicle placode-specific gene signature obtained from microarray analysis of sorted EGFP-positive keratinocytes at E14.5. Genes mentioned in the text are underlined. Functional categories with representative examples of mRNAs up-regulated ≥ 1.5x are listed. Fold differences between placodes and interfollicular epidermis (EGFP-negative) are indicated in parentheses. Genes highlighted in red were down-regulated and in blue up-regulated in Δ*N*-positive epidermal keratinocytes. Genes in black were specifically enriched in hair follicle placodes, but not regulated by NF-κB in a significant manner. Note that *Fn1* (in light red) expression is enriched 5x in hair follicle placodes, but is only weakly down-regulated in Δ*N* versus controls (~1.3x). **(D)** Quantitative real-time PCR (qRT-PCR) analysis using either RNA samples from epidermal keratinocytes of control or Δ*N* embryos at E14.5 (left chart), or from FACS-sorted EGFP-positive (HF) or EGFP-negative (IFE) keratinocytes of *κ-EGFP* embryos at E14.5 (right chart). Statistical analyses were performed using a two-tailed unpaired t-test. Data are presented as mean±s.e.m. * P<0.05; ** P<0.01; ***, P<0.001. **(E)** In situ hybridization for indicated mRNA probes using sagittal skin sections of control and Δ*N* embryos at E14.5. Scale bars: 50 μm.

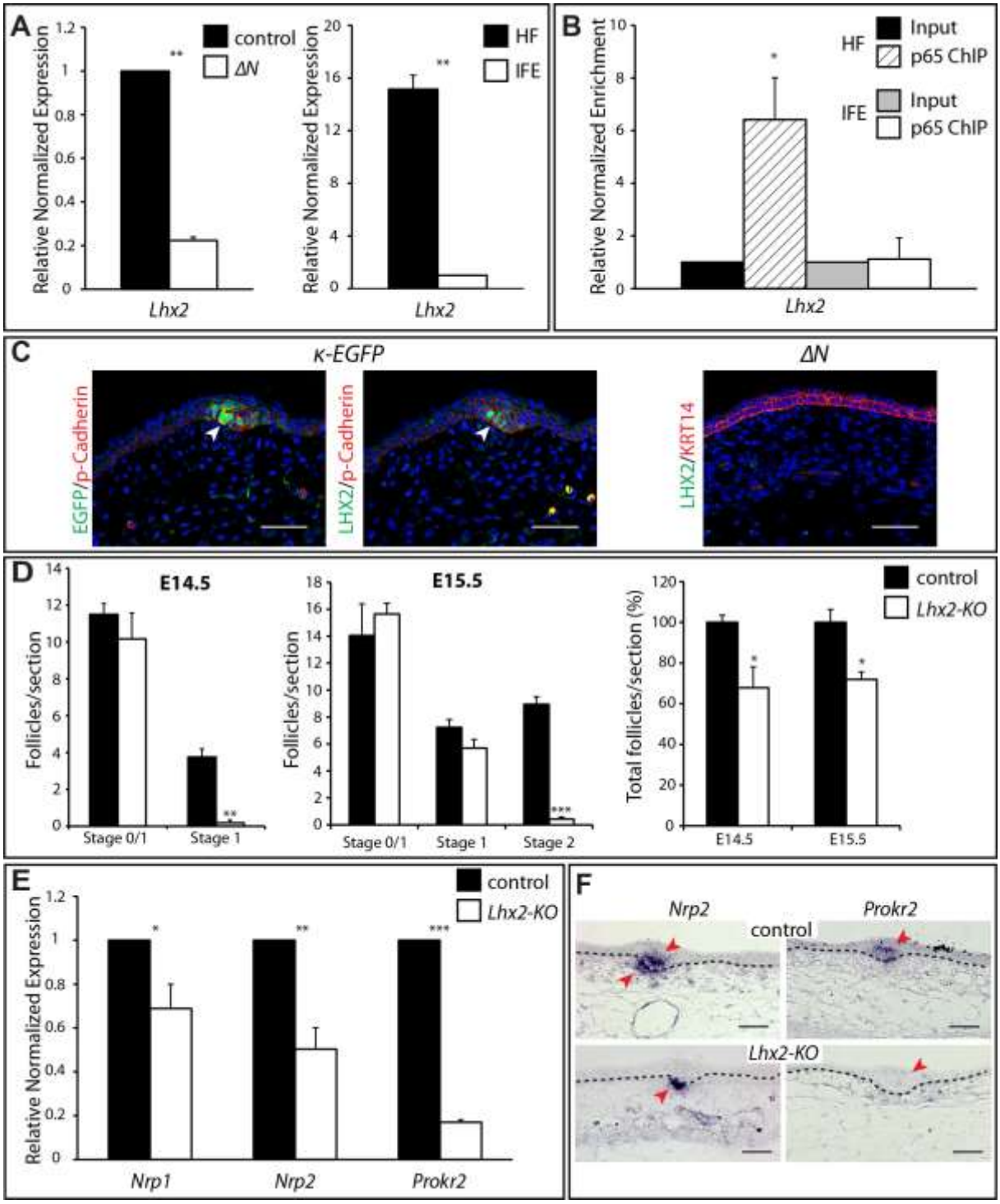

Fig. 2 *Lhx2* is a direct target gene of NF-κB during primary placode formation and acts in concert with NF-κB to regulate genes involved in cell migration. (A) Quantitative real-time PCR analysis (qRT-PCR) of *Lhx2* mRNA expression from epidermal keratinocytes of control or Δ*N*

embryos (left chart), or from EGFP-positive placode and EGFP-negative IFE keratinocytes (right chart) at E14.5. **(B)** NF-κB p65-specific chromatin immunoprecipitation (ChIP) assays using EGFP-positive placode and EGFP-negative IFE keratinocyte extracts from *κ-EGFP* embryos at E14.5, and *Lhx2* and control *Gapdh* primers. **(C)** Immunostaining on serial sagittal back skin sections of *κ-EGFP* and Δ*N* mice at E14.5 using antibodies against EGFP, P-Cadherin, LHX2 and KRT14. **(D)** Analysis of primary HF development in *Lhx2*-KO mice revealed a dramatic reduction of stage 2 primary HF at E15.5 (left chart). Overall primary HF density in *Lhx2*-KO embryos at E15.5 is reduced by ~30% (right chart). The graphs provide quantification from multiple back skin sections of three biological replicates. **(E)** Quantitative real-time PCR analysis (qRT-PCR) for selected NF-κB target genes involved in cell migration using mRNA isolated from epidermal keratinocytes of either *Lhx2*-KO or control embryos at E14.5. **(F)** In situ hybridization for *Nrp2* and *Prokr2* mRNA on sagittal skin sections of control and *Lhx2*-KO embryos at E14.5. Scale bars: 50 μm. All statistical analyses (**A**, **B**, **D** and **E**) were performed using two-tailed unpaired t-test. Data are presented as mean±s.e.m. * $P<0.05$; ** $P<0.01$; ***, $P<0.001$.

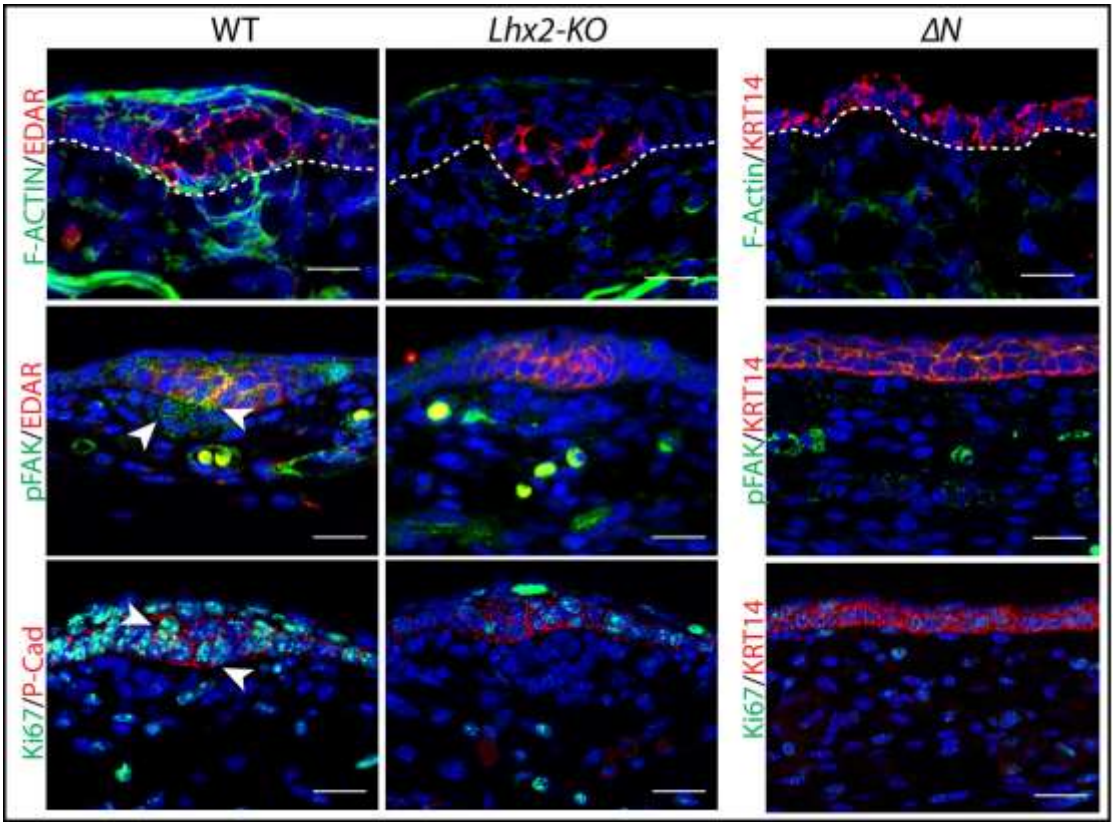

Fig. 3 Δ*N* and *Lhx2*-KO mice exhibit keratinocyte migratory and proliferative defects in the down-growing primary HF placode. Immunostaining on sagittal sections of control, *Lhx2*-KO and Δ*N* embryos at E14.5. Cytoskeletal organization and dynamics (F-Actin/Phalloidin), and phosphorylation of FAK (pFAK) were used as markers for cell migration. As readout for proliferation Ki67 expression was used. Antibodies against P-cadherin and EDAR (both red) were used to differentiate hair follicle placodes. Scale bars: 20 µm.

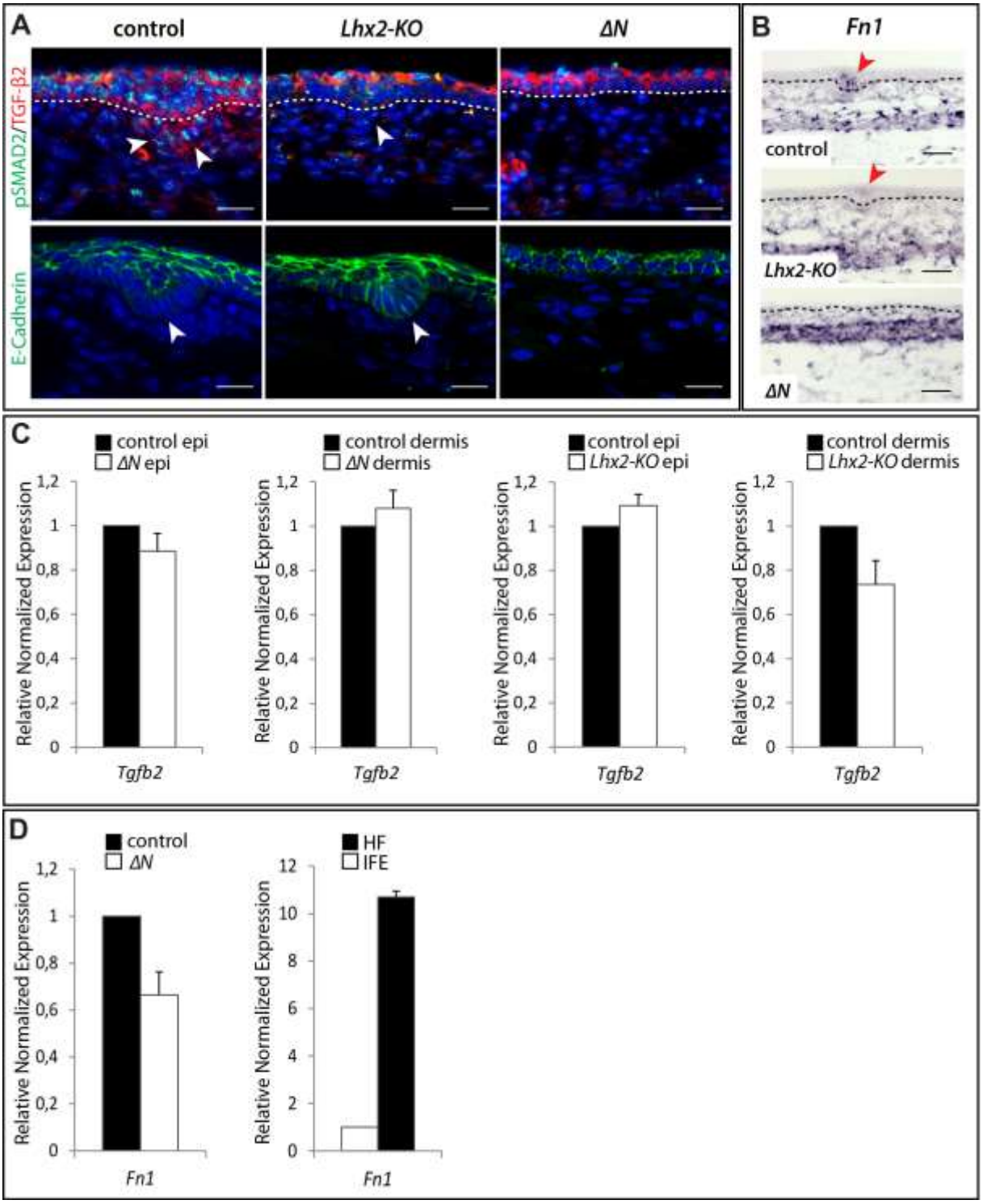

Fig. 4 *Lhx2*-KO mice exhibit delayed primary HF development and, as in Δ*N* mice, impaired TGF-β signaling and lack of E-cadherin down-regulation. (A) Immunostaining on sagittal sections of control, *Lhx2*-KO and Δ*N* embryos at E14.5 using antibodies against anti-pSMAD2 (green), TGFβ2

(red) and E-cadherin (green). **(B)** In situ hybridization for *Fn1* mRNA on sagittal sections of control, *Lhx2*-KO and Δ*N* embryos at E14.5. Scale bars: 20 µm (immunofluorescence), 50 µm (in situ hybridization). **(C, D)** Quantitative real-time PCR analysis (qRT-PCR) for *Tgfb2* **(C)** and *Fn1* **(D)** using mRNA isolated from epidermal keratinocytes of either Δ*N*, *Lhx2*-KO or control embryos at E14.5.

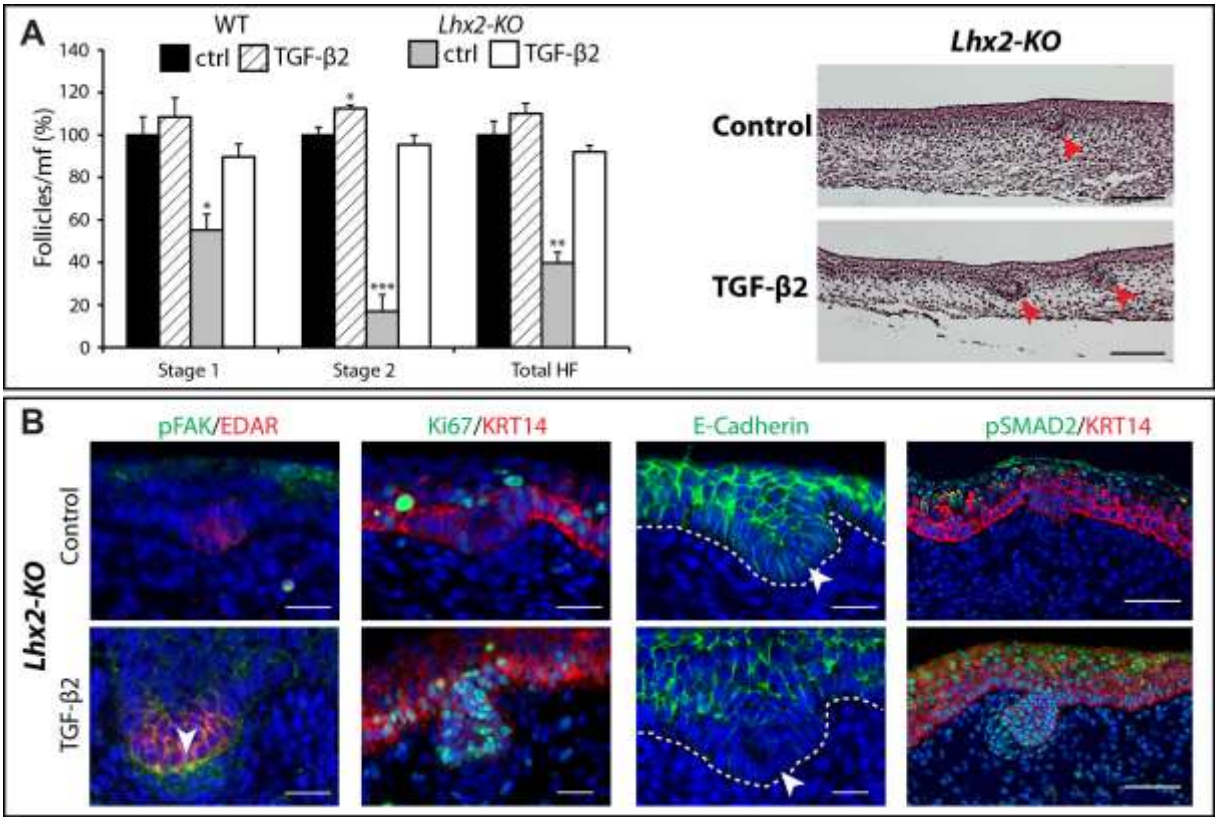

Fig. 5 Treatment of *Lhx2*-KO embryonic skin explants with TGFβ2 rescued proper primary HF development. (A) Embryonic skin explants of E14.5 control or *Lhx2*-KO mice were either left untreated (ctrl) or treated with recombinant TGFβ2 (100 ng/ml) for 24 hrs. Skin samples were stained with H&E (left panel), and follicles were quantified in percent/microscopic field (right panel). Statistical analyses were performed using two-tailed unpaired t-test. Data were pooled from three biological replicates and presented as mean±s.e.m. * P<0.05; ** P<0.01; ***, P<0.001. Scale bars: 100 µm **(B)** Immunostaining on *Lhx2*-KO explants treated with recombinant TGFβ2 or left untreated using antibodies against pFAK, EDAR, Ki67, KRT14, E-cadherin and pSMAD2. Scale bars: 20 µm / 50 µm (pSMAD2).

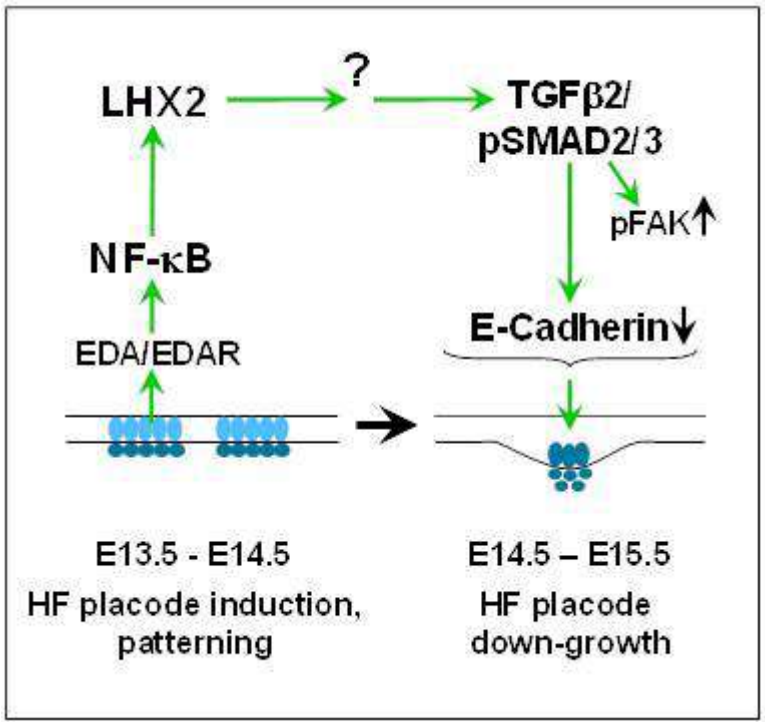

Fig. 6 Model for primary placode down-growth involving NF-κB signaling. We show here that LHX2 expression is directly regulated by EDA-A1/EDAR/NF-κB signaling, as NF-κB activity in primary placodes depends on EDA-A1/EDAR (Schmidt-Ullrich et al., 2006). By a yet unknown mechanism LHX2 activates TGFβ2 signaling in primary hair follicle placodes which results in phosphorylation of FAK (pFAK), and, importantly, in down-regulation of E-Cadherin expression. Together these results introduce a novel NF-κB-LHX2-TGFβ2 signaling axis which is required for establishing the proper conditions for placode down-growth, and may also be relevant for other epidermal-mesenchymal tissue interactions, for EMT or for tumor growth.

REFERENCES

Ahtiainen, L., Lefebvre, S., Lindfors, P. H., Renvoise, E., Shirokova, V., Vartiainen, M. K., Thesleff, I. and Mikkola, M. L. (2014). Directional cell migration, but not proliferation, drives hair placode morphogenesis. *Dev Cell* 28, 588-602.

Andl, T., Reddy, S. T., Gaddapara, T. and Millar, S. E. (2002). WNT signals are required for the initiation of hair follicle development. *Dev Cell* 2, 643-53.

Bazzi, H., Fantauzzo, K. A., Richardson, G. D., Jahoda, C. A. and Christiano, A. M. (2007). The Wnt inhibitor, Dickkopf 4, is induced by canonical Wnt signaling during ectodermal appendage morphogenesis. *Dev Biol* 305, 498 - 507.

Biggs, L. C. and Mikkola, M. L. (2014). Early inductive events in ectodermal appendage morphogenesis. *Semin Cell Dev Biol* 25-26, 11-21.

Botchkarev, V. A., Botchkareva, N. V., Roth, W., Nakamura, M., Chen, L. H., Herzog, W., Lindner, G., McMahon, J. A., Peters, C., Lauster, R. et al. (1999). Noggin is a mesenchymally derived stimulator of hair-follicle induction. *Nat Cell Biol* 1, 158-64.

Botchkareva, N. V., Botchkarev, V. A., Chen, L. H., Lindner, G. and Paus, R. (1999). A role for p75 neurotrophin receptor in the control of hair follicle morphogenesis. *Dev Biol* 216, 135-53.

Bulchand, S., Grove, E. A., Porter, F. D. and Tole, S. (2001). LIM-homeodomain gene Lhx2 regulates the formation of the cortical hem. *Mech Dev* 100, 165-75.

Chen, D., Jarrell, A., Guo, C., Lang, R. and Atit, R. (2012). Dermal beta-catenin activity in response to epidermal Wnt ligands is required for fibroblast proliferation and hair follicle initiation. *Development* 139, 1522-33.

Chiang, C., Swan, R. Z., Grachtchouk, M., Bolinger, M., Litingtung, Y., Robertson, E. K., Cooper, M. K., Gaffield, W., Westphal, H., Beachy, P. A. et al. (1999). Essential role for Sonic hedgehog during hair follicle morphogenesis. *Dev Biol* 205, 1-9.

Cui, C. Y., Durmowicz, M., Tanaka, T. S., Hartung, A. J., Tezuka, T., Hashimoto, K., Ko, M. S., Srivastava, A. K. and 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, 1763-73.

Cui, C. Y., Hashimoto, T., Grivennikov, S. I., Piao, Y., Nedospasov, S. A. and Schlessinger, D. (2006). Ectodysplasin regulates the lymphotoxin-beta pathway for hair differentiation. *Proc Natl Acad Sci U S A* 103, 9142-7.

Essayem, S., Kovacic-Milivojevic, B., Baumbusch, C., McDonagh, S., Dolganov, G., Howerton, K., Larocque, N., Mauro, T., Ramirez, A., Ramos, D. M. et al. (2006). Hair cycle and wound healing in mice with a keratinocyte-restricted deletion of FAK. *Oncogene* 25, 1081-9.

Falconer, D. S. (1952). A totally sex-linked gene in the house mouse. *Nature* 169, 664-5.

Fantauzzo, K. A., Bazzi, H., Jahoda, C. A. and Christiano, A. M. (2008a). Dynamic expression of the zinc-finger transcription factor Trps1 during hair follicle morphogenesis and cycling. *Gene Expr Patterns* 8, 51-7.

Fantauzzo, K. A., Tadin-Strapps, M., You, Y., Mentzer, S. E., Baumeister, F. A., Cianfarani, S., Van Maldergem, L., Warburton, D., Sundberg, J. P. and Christiano, A. M. (2008b). A position effect on TRPS1 is associated with Ambras syndrome in humans and the Koala phenotype in mice. *Hum Mol Genet* 17, 3539-51.

Fliniaux, I., Mikkola, M. L., Lefebvre, S. and Thesleff, I. (2008). Identification of dkk4 as a target of Eda-A1/Edar pathway reveals an unexpected role of ectodysplasin as inhibitor of Wnt signalling in ectodermal placodes. *Dev Biol* 320, 60-71.

Foitzik, K., Paus, R., Doetschman, T. and Dotto, G. P. (1999). The TGF-beta2 isoform is both a required and sufficient inducer of murine hair follicle morphogenesis. *Dev Biol* 212, 278-89.

Folgueras, A. R., Guo, X., Pasolli, H. A., Stokes, N., Polak, L., Zheng, D. and Fuchs, E. (2013). Architectural niche organization by LHX2 is linked to hair follicle stem cell function. *Cell Stem Cell* 13, 314-27.

Frame, M. C., Patel, H., Serrels, B., Lietha, D. and Eck, M. J. (2010). The FERM domain: organizing the structure and function of FAK. *Nat Rev Mol Cell Biol* 11, 802-14.

Fuchs, E. (2007). Scratching the surface of skin development. *Nature.* 445, 834-42.

Glinka, Y., Stoilova, S., Mohammed, N. and Prud'homme, G. J. (2011). Neuropilin-1 exerts co-receptor function for TGF-beta-1 on the membrane of cancer cells and enhances responses to both latent and active TGF-beta. *Carcinogenesis* 32, 613-21.

Hardy, M. H. (1992). The secret life of the hair follicle. *Trends Genet* 8, 55-61.

Headon, D. J. and Overbeek, P. A. (1999). Involvement of a novel Tnf receptor homologue in hair follicle induction [see comments]. *Nat Genet* 22, 370-4.

Hirota, J. and Mombaerts, P. (2004). The LIM-homeodomain protein Lhx2 is required for complete development of mouse olfactory sensory neurons. *Proc Natl Acad Sci U S A* 101, 8751-5.

Jamora, C., DasGupta, R., Kocieniewski, P. and Fuchs, E. (2003). Links between signal transduction, transcription and adhesion in epithelial bud development. *Nature* 422, 317-22.

Jamora, C., Lee, P., Kocieniewski, P., Azhar, M., Hosokawa, R., Chai, Y. and Fuchs, E. (2005). A signaling pathway involving TGF-beta2 and snail in hair follicle morphogenesis. *PLoS Biol* 3, e11.

Jiang, T. X., Widelitz, R. B., Shen, W. M., Will, P., Wu, D. Y., Lin, C. M., Jung, H. S. and Chuong, C. M. (2004). Integument pattern formation involves genetic and epigenetic controls: feather arrays simulated by digital hormone models. *Int J Dev Biol* 48, 117-35.

Johnson, J. L., Hall, T. E., Dyson, J. M., Sonntag, C., Ayers, K., Berger, S., Gautier, P., Mitchell, C., Hollway, G. E. and Currie, P. D. (2012). Scube activity is necessary for Hedgehog signal transduction in vivo. *Dev Biol* 368, 193-202.

Kadaja, M., Keyes, B. E., Lin, M., Pasolli, H. A., Genander, M., Polak, L., Stokes, N., Zheng, D. and Fuchs, E. (2014). SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. *Genes Dev* 28, 328-41.

Kere, J., Srivastava, A. K., Montonen, O., Zonana, J., Thomas, N., Ferguson, B., Munoz, F., Morgan, D., Clarke, A., Baybayan, P. et al. (1996). X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. *Nat Genet* 13, 409-16.

Kiso, M., Tanaka, S., Saba, R., Matsuda, S., Shimizu, A., Ohyama, M., Okano, H. J., Shiroishi, T., Okano, H. and Saga, Y. (2009). The disruption of Sox21-mediated hair shaft cuticle differentiation causes cyclic alopecia in mice. *Proc Natl Acad Sci U S A* 106, 9292-7.

Kumar, A., Eby, M. T., Sinha, S., Jasmin, A. and Chaudhary, P. M. (2001). The ectodermal dysplasia receptor activates the nuclear factor-kappaB, JNK, and cell death pathways and binds to ectodysplasin A. *J Biol Chem* 276, 2668-77.

Kunath, M., Ludecke, H. J. and Vortkamp, A. (2002). Expression of Trps1 during mouse embryonic development. *Gene Expr Patterns* 2, 119-22.

Laurikkala, J., Pispa, J., Jung, H. S., Nieminen, P., Mikkola, M., Wang, X., Saarialho-Kere, U., Galceran, J., Grosschedl, R. and Thesleff, I. (2002). Regulation of hair follicle development by the TNF signal ectodysplasin and its receptor Edar. *Development* 129, 2541-53.

Le Bail, O., Schmidt-Ullrich, R. and Israel, A. (1993). Promoter analysis of the gene encoding the I kappa B-alpha/MAD3 inhibitor of NF-kappa B: positive regulation by members of the rel/NF-kappa B family. *EMBO J* 12, 5043-9.

Lefebvre, S., Fliniaux, I., Schneider, P. and Mikkola, M. L. (2012). Identification of ectodysplasin target genes reveals the involvement of chemokines in hair development. *J Invest Dermatol* 132, 1094-102.
Lettice, L. A., Williamson, I., Wiltshire, J. H., Peluso, S., Devenney, P. S., Hill, A. E., Essafi, A., Hagman, J., Mort, R., Grimes, G. et al. (2012). Opposing functions of the ETS factor family define Shh spatial expression in limb buds and underlie polydactyly. *Dev Cell* 22, 459-67.
Malik, T. H., Von Stechow, D., Bronson, R. T. and Shivdasani, R. A. (2002). Deletion of the GATA domain of TRPS1 causes an absence of facial hair and provides new insights into the bone disorder in inherited tricho-rhino-phalangeal syndromes. *Mol Cell Biol* 22, 8592-600.
Mangale, V. S., Hirokawa, K. E., Satyaki, P. R., Gokulchandran, N., Chikbire, S., Subramanian, L., Shetty, A. S., Martynoga, B., Paul, J., Mai, M. V. et al. (2008). Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. *Science* 319, 304-9.
Mao, J., McGlinn, E., Huang, P., Tabin, C. J. and McMahon, A. P. (2009). Fgf-dependent Etv4/5 activity is required for posterior restriction of Sonic Hedgehog and promoting outgrowth of the vertebrate limb. *Dev Cell* 16, 600-6.
Mardaryev, A. N., Meier, N., Poterlowicz, K., Sharov, A. A., Sharova, T. Y., Ahmed, M. I., Rapisarda, V., Lewis, C., Fessing, M. Y., Ruenger, T. M. et al. (2011). Lhx2 differentially regulates Sox9, Tcf4 and Lgr5 in hair follicle stem cells to promote epidermal regeneration after injury. *Development* 138, 4843-52.
Mill, P., Mo, R., Fu, H., Grachtchouk, M., Kim, P. C., Dlugosz, A. A. and Hui, C. C. (2003). Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev* 17, 282-94.
Momeni, P., Glockner, G., Schmidt, O., von Holtum, D., Albrecht, B., Gillessen-Kaesbach, G., Hennekam, R., Meinecke, P., Zabel, B., Rosenthal, A. et al. (2000). Mutations in a new gene, encoding a zinc-finger protein, cause tricho-rhino-phalangeal syndrome type I. *Nat Genet* 24, 71-4.
Mou, C., Jackson, B., Schneider, P., Overbeek, P. A. and Headon, D. J. (2006). Generation of the primary hair follicle pattern. *Proc Natl Acad Sci U S A.* 103, 9075-80. Epub 2006 Jun 12.
Mustonen, T., Pispa, J., Mikkola, M. L., Pummila, M., Kangas, A. T., Pakkasjarvi, L., Jaatinen, R. and Thesleff, I. (2003). Stimulation of ectodermal organ development by Ectodysplasin-A1. *Dev Biol* 259, 123-36.
Nowak, J. A., Polak, L., Pasolli, H. A. and Fuchs, E. (2008). Hair follicle stem cells are specified and function in early skin morphogenesis. *Cell Stem Cell* 3, 33-43.
Oro, A. E. and Scott, M. P. (1998). Splitting hairs: dissecting roles of signaling systems in epidermal development. *Cell* 95, 575-8.
Paus, R., Muller-Rover, S., Van Der Veen, C., Maurer, M., Eichmuller, S., Ling, G., Hofmann, U., Foitzik, K., Mecklenburg, L. and Handjiski, B. (1999). A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *J Invest Dermatol* 113, 523-32.
Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D. et al. (1997). Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* 124, 2935-44.
Pummila, M., Fliniaux, I., Jaatinen, R., James, M. J., Laurikkala, J., Schneider, P., Thesleff, I. and Mikkola, M. L. (2007). Ectodysplasin has a dual role in ectodermal organogenesis: inhibition of Bmp activity and induction of Shh expression. *Development.* 134, 117-25.

Rhee, H., Polak, L. and Fuchs, E. (2006). Lhx2 maintains stem cell character in hair follicles. *Science* 312, 1946-9.
Schaller, M. D. (2010). Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci* 123, 1007-13.
Schmidt-Ullrich, R., Aebischer, T., Hulsken, J., Birchmeier, W., Klemm, U. and Scheidereit, C. (2001). Requirement of NF-kappaB/Rel for the development of hair follicles and other epidermal appendices. *Development* 128, 3843-3853.
Schmidt-Ullrich, R., Memet, S., Lilienbaum, A., Feuillard, J., Raphael, M. and Israel, A. (1996). NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity. *Development* 122, 2117-28.
Schmidt-Ullrich, R. and Paus, R. (2005). Molecular principles of hair follicle induction and morphogenesis. *Bioessays* 27, 247-61.
Schmidt-Ullrich, R., Tobin, D. J., Lenhard, D., Schneider, P., Paus, R. and Scheidereit, C. (2006). NF-kappaB transmits Eda A1/EdaR signalling to activate Shh and cyclin D1 expression, and controls post-initiation hair placode down growth. *Development* 133, 1045-57.
Schneider, M. R., Schmidt-Ullrich, R. and Paus, R. (2009). The hair follicle as a dynamic miniorgan. *Curr Biol* 19, R132-42.
Schober, M., Raghavan, S., Nikolova, M., Polak, L., Pasolli, H. A., Beggs, H. E., Reichardt, L. F. and Fuchs, E. (2007). Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol* 176, 667-80.
Schwarzbauer, J. E. and DeSimone, D. W. (2011). Fibronectins, their fibrillogenesis, and in vivo functions. *Cold Spring Harb Perspect Biol* 3.
Sennett, R. and Rendl, M. (2012). Mesenchymal-epithelial interactions during hair follicle morphogenesis and cycling. *Semin Cell Dev Biol* 23, 917-27.
Sennett, R., Wang, Z., Rezza, A., Grisanti, L., Roitershtein, N., Sicchio, C., Mok, K. W., Heitman, N. J., Clavel, C., Ma'ayan, A. et al. (2015). An Integrated Transcriptome Atlas of Embryonic Hair Follicle Progenitors, Their Niche, and the Developing Skin. *Dev Cell*.
Shetty, A. S., Godbole, G., Maheshwari, U., Padmanabhan, H., Chaudhary, R., Muralidharan, B., Hou, P. S., Monuki, E. S., Kuo, H. C., Rema, V. et al. (2013). Lhx2 regulates a cortex-specific mechanism for barrel formation. *Proc Natl Acad Sci U S A* 110, E4913-21.
Shirokova, V., Jussila, M., Hytonen, M. K., Perala, N., Drogemuller, C., Leeb, T., Lohi, H., Sainio, K., Thesleff, I. and Mikkola, M. L. (2013). Expression of Foxi3 is regulated by ectodysplasin in skin appendage placodes. *Dev Dyn* 242, 593-603.
Sick, S., Reinker, S., Timmer, J. and Schlake, T. (2006). WNT and DKK determine hair follicle spacing through a reaction-diffusion mechanism. *Science.* 314, 1447-50. Epub 2006 Nov 2.
Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H. and Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* 2, 249-56.
St-Jacques, B., Dassule, H. R., Karavanova, I., Botchkarev, V. A., Li, J., Danielian, P. S., McMahon, J. A., Lewis, P. M., Paus, R. and McMahon, A. P. (1998). Sonic hedgehog signaling is essential for hair development. *Curr Biol* 8, 1058-68.
Sulzmaier, F. J., Jean, C. and Schlaepfer, D. D. (2014). FAK in cancer: mechanistic findings and clinical applications. *Nat Rev Cancer* 14, 598-610.
Takeuchi, M. and Baichwal, V. R. (1995). Induction of the gene encoding mucosal vascular addressin cell adhesion molecule 1 by tumor necrosis factor alpha is mediated by NF-kappa B proteins. *Proc Natl Acad Sci U S A* 92, 3561-5.
Tornqvist, G., Sandberg, A., Hagglund, A. C. and Carlsson, L. (2010). Cyclic expression of lhx2 regulates hair formation. *PLoS Genet* 6, e1000904.

Tsao, K. C., Tu, C. F., Lee, S. J. and Yang, R. B. (2013). Zebrafish scube1 (signal peptide-CUB (complement protein C1r/C1s, Uegf, and Bmp1)-EGF (epidermal growth factor) domain-containing protein 1) is involved in primitive hematopoiesis. *J Biol Chem* 288, 5017-26.

Vidal, V. P., Chaboissier, M. C., Lutzkendorf, S., Cotsarelis, G., Mill, P., Hui, C. C., Ortonne, N., Ortonne, J. P. and Schedl, A. (2005). Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. *Curr Biol* 15, 1340-51.

Worthington, J. J., Klementowicz, J. E. and Travis, M. A. (2011). TGFbeta: a sleeping giant awoken by integrins. *Trends Biochem Sci* 36, 47-54.

Yan, M., Wang, L. C., Hymowitz, S. G., Schilbach, S., Lee, J., Goddard, A., de Vos, A. M., Gao, W. Q. and Dixit, V. M. (2000). Two-amino acid molecular switch in an epithelial morphogen that regulates binding to two distinct receptors. *Science* 290, 523-7.

Yoon, S., Woo, S. U., Kang, J. H., Kim, K., Shin, H. J., Gwak, H. S., Park, S. and Chwae, Y. J. (2012). NF-kappaB and STAT3 cooperatively induce IL6 in starved cancer cells. *Oncogene* 31, 3467-81.

Yoshizaki, T., Sato, H., Furukawa, M. and Pagano, J. S. (1998). The expression of matrix metalloproteinase 9 is enhanced by Epstein-Barr virus latent membrane protein 1. *Proc Natl Acad Sci U S A* 95, 3621-6.

Zhang, Y., Andl, T., Yang, S. H., Teta, M., Liu, F., Seykora, J. T., Tobias, J. W., Piccolo, S., Schmidt-Ullrich, R., Nagy, A. et al. (2008). Activation of {beta}-catenin signaling programs embryonic epidermis to hair follicle fate. *Development* 135, 2161 - 2172.

Zhang, Y., Tomann, P., Andl, T., Gallant, N. M., Huelsken, J., Jerchow, B., Birchmeier, W., Paus, R., Piccolo, S., Mikkola, M. L. et al. (2009a). Reciprocal requirements for EDA/EDAR/NF-kappaB and Wnt/beta-catenin signaling pathways in hair follicle induction. *Dev Cell* 17, 49-61.

Zhang, Z., Verheyden, J. M., Hassell, J. A. and Sun, X. (2009b). FGF-regulated Etv genes are essential for repressing Shh expression in mouse limb buds. *Dev Cell* 16, 607-13.