Role of Insulin/IGF-1-regulated FoxO transcription factors in epidermal morphogenesis

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Abstract

The mammalian epidermis is a self-renewing protective epithelial barrier against external challenges and dehydration, which is formed during embryogenesis through a stratification program. The external signals that initiate and regulate this program are presently unknown. Previous findings in the laboratory identified epidermal insulin/IGF-1 signaling (IIS) as key regulators of epidermal morphogenesis. Mice with an epidermal deletion of either the insulin receptor, the IGF-1 receptor or both showed a increasing reduction in the formation of suprabasal layers, impaired proliferative potential with a temporary arrest in mitosis. The goal of this thesis was to identify how epidermal IIS controls self-renewal and stratification during embryogenesis and address the role of the IIS controlled Forkhead box-O (FoxO) transcription factors, in these processes. The results show that IIS signaling is activated in mitosis and sufficient to drive mitotic progression. Initiation of stratification is accompanied by a shift from symmetric (SCD) to asymmetric division (ACD). This shift is impaired upon loss of IIS as a result of a biased loss of ACDs. We further identified the transcription factor p63 as a downstream signaling target of IIS. P63 is a master regulator of epidermal specification, controls the shift towards ACDs and promotes proliferative potential. Upon loss of IIS, FoxO transcription factors were retained in the nucleus where they bind and inhibited p63-regulated transcription, which was independent of direct FoxO DNA binding. Small interfering-RNA mediated knockdown of FoxOs reversed IIS loss induced alterations in p63 target gene expression. Accordingly, transgenic expression of a constitutive nuclear FoxO variant in mice epidermis abrogates ACD, inhibits p63-regulated transcription and stratification, mimicking loss of p63. In summary, this study revealed a critical role for IIS-dependent control of p63 activity in coordination of ACD and stratification during epithelial morphogenesis.

Zusammenfassung

Die Epidermis von Säugetieren bildet eine sich selbst erneuernde Schutzbarriere gegen äußere Einflüsse und Dehydrierung, die während der Embryonalentwicklung durch Stratifizierung entsteht. Die äußeren Signale, die dieses Programm initiieren und regulieren sind momentan noch nicht bekannt. Vorrangegangene Laborergebnisse haben gezeigt dass Insulin/IGF-1 wichtige Regulatoren der epidermalen Morphogenese sind. Mäuse denen der Insulinrezeptor (IR), der IGF-1 Rezeptor (IGF-1R) oder beide Rezeptoren gleichzeitig in der Epidermis fehlten, entwickelten weniger suprabasale Schichten und hatten ein verringertes proliferatives Potential mit vorrübergehendem Mitosearrest. Ziel dieser Arbeit war es nun zu verstehen, wie epidermales IIS Selbsterneuerung und Stratifizierung während der Embryogenese kontrolliert und zu fragen welche Rolle Forkhead box-O (FoxO) Transkriptionsfaktoren dabei spielen. Unsere Ergebnisse zeigten dass IIS während der Mitose aktiv ist und die Mitose fortführen konnte. Während der Initiierung der Stratifizierung findet ein Umschalten von symmetrischen (SCD) zu asymmetrischen Zellteilungen (ACD) statt. Diese Verschiebung wurde durch den Verlust von IIS gestört und resultierte in weniger ACDs. Des weiteren identifizierten wir den Transkriptionsfaktor p63 als nachgelagertes Regulationsziel von IIS. P63, der Hauptregulierer der epidermalen Spezifizierung, kontrolliert diese Umverteilung zu ACDs und das proliferative Potential der Zellen. Durch den Verlust von IIS wird FoxO im Nukleus zurückgehalten, bindet und hemmt dort die p63 regulierte Transkription unabhängig von einer direkten FoxO-DNA Interaktion. SiRNA vermittelter knockdown von FoxO konnte den Verlust von IIS auf die p63 Zielgenexpression umkehren. Wie erwartet, führte die transgene Expression einer konstitutiv nukleären FoxO Variante in der Mausepidermis zum Verlust von ACDs, inhibierte die p63-regulierte Transkription und Stratifizierung, ähnlich dem Verlust von p63. Diese Studie konnte eine wichtige Funktion für die IIS-abhängige Kontrolle von p63 bei der Koordination von ACDs und Stratifizierung aufzeigen.

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1. Introduction

1.1. The mammalian epidermis

The mammalian skin is composed of two distinct compartments, the dermis and the epidermis, which are separated by a basement membrane. The outermost layer, the epidermis, is a stratifying epithelium that forms a protective barrier against external challenges and dehydration (Fig. 1). This life-long self-renewing tissue is composed of the interfollicular epidermis (IFE) and its appendages, the hair follicles, sebaceous glands and sweat glands (Chuong et al., 2000; Fuchs et al., 2002; Watt et al., 2013). The majority of the cells in the epidermis are keratinocytes but also other cell types, e.g. dendritic cells, macrophages or melanocytes, are present. The IFE is organized in distinct layers beginning with the basal cell layer (*stratum basale*), which through cell adhesion receptors such as integrins adhere to the underlying basement membrane. The keratinocytes in this layer are undifferentiated, have a high proliferative potential and are characterized by the expression of the cytoskeletal intermediate filaments keratin 14 and keratin 5 (K5 and K14) (Nelson and Sun, 1983; Eichner et al., 1986; Koster and Roop, 2007). Due to yet ill-defined stimuli certain cells of the basal layer move outwards into the suprabasal spinous layer (*stratum spinosum*) with post-mitotic cells that undergo further differentiation (Smart, 1970). This layer is mainly characterized by the stratification markers keratin 1 and keratin 10 (K1 and K10). These spinous cells undergo terminal differentiation and form the granular layer (*stratum granulosum*) and the subsequent cornified layer (*stratum corneum*). This final process is accompanied by the initiation of the late stratification markers loricrine and involucrin and involves the formation of the epidermal barrier (Bickenbach et al., 1995). A major component of the granular layer are the intercellular tight junctions, which are ion and size selective paracellular diffusion barriers that prevent diffusion of solutes through the intercellular space and thereby

form the paracellular diffusion barrier (Furuse et al., 2002; Tunggal et al., 2005); Morita et al., 2011). The dead cornified layer consists of corneocytes that together with extracellular lipids forms the outer most barrier. During the final terminal differentiation step cells flatten, degrade their DNA and subsequently assemble the cornified envelope underneath the plasma membrane by the incorporation and processing of precursor proteins, such as filaggrins and small proline-rich proteins (Sprr's) (Segre, 2003). Finally, the cells become more permeable and the influx of calcium activates transglutaminases that crosslink proteins of the cornified envelope, which forms a scaffold for the lipids that are produced by the layers underneath (Segre, 2003).

Although essential structural components of the epidermis are identified, the precise regulation of the balance between proliferation and differentiation during epidermal homeostasis to maintain the barrier needs further investigation. For example, it is poorly understood which signals are important for the initiation of this stratified epithelium and how these signals are altered during epidermal pathogenesis.

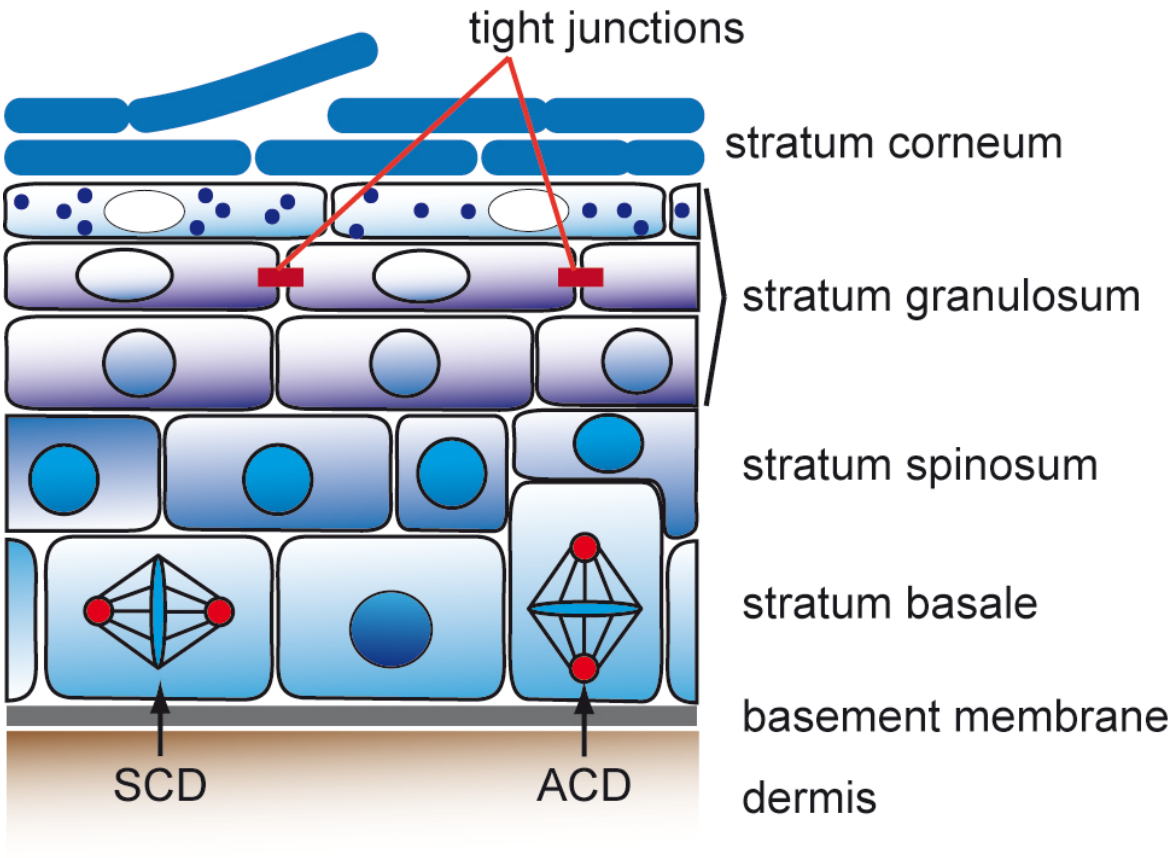


Figure 1: Schematic representation of the mammalian interfollicular epidermis. The interfollicular epidermis (IFE) is a stratified epithelium with different layers. The basal layer (*stratum basale*) contains proliferating keratinocytes. Cells in this layer can either divide symmetrically expanding the basal layer or asymmetrically resulting in one basal and one suprabasal daughter. Upon differentiation keratinocytes migrate into the spinous layer (*stratum spinosum*). In the granular layer (*stratum granulosum*), the cells are more flattened and form the tight junction barrier. The *stratum corneum* forms the most outer layer of the epidermis and fuctions as a the lipid barrier. SCD- symmetric cell division, ACD-asymmetric cell division

1.2. Epidermal morphogenesis

The barrier function of the epidermis is established during embryogenesis as a result of a precisely coordinated stratification program (Koster and Roop, 2007) (Fig. 2). In mice, the execution of this program initiates at around embryonic day 8.5 (E8.5) when cells of the ectoderm are committed to an epidermal fate and start to express the fibrous structural proteins K5 and K14 (Byrne et al., 1994). This initial step of stratification is controlled by p63, a member of the p53 transcription factor family, which specifies the epidermal lineage (Green et al., 2003) by blocking neural specification in the surface ectoderm (Bakkers et al., 2002). Subsequently, at E9.5 the first newly formed layer of keratinocytes gives rise to the periderm, a specialized simple epithelium that forms a barrier towards placental fluids and is shed off before birth, when the functional barrier of the epidermis is formed (M'Boneko and Merker, 1988). At around embryonic day 12.5 (E12.5) stratification is initiated and the epidermis becomes multilayered resulting in the formation of the intermediate spinous layer between the basal layer and the periderm (Smart, 1970; Weiss and Zelickson, 1975). In this layer the keratinocytes are committed to differentiation, which is accompanied by the expression of K1 and K10. How this cell layer is initiated and maintained is not completely understood. The prevailing model suggests that a change in the plane of cell division in the basal keratinocytes, switching from parallel to perpendicular to the basement membrane, is important for this process (Smart, 1970; Lechler and Fuchs, 2005). Moreover, this division in the cells of the basal layer is associated with the asymmetric distribution of proteins, which have been implicated in asymmetric cell divisions (ACD) in lower organisms (Lechler and Fuchs, 2005; Poulson and Lechler, 2010) Williams et al., 2011). In lower organisms, this asymmetric distribution of cell fate determinants is responsible for the development of daughter cells with two distinct cell fates. In the epidermis, this

perpendicular or asymmetric division results in one daughter cell that remains in the basal layer and contributes to the maintenance of this layer and the other daughter cell is committed towards differentiation and thereby contributes to suprabasal layers (see also section 1.2.1).

During the transition from E15.5 to E16.5 the suprabasal layers expand from 2-3 to 4-6 layers accompanied by the formation of the granular layer and the initiation of *stratum corneum* formation at the dorsal site resulting in local barrier function (Byrne et al., 1994). At this step a program for the expression of essential proteins for barrier formation and terminal differentiation, such as loricrin and filaggrin, is initiated (Bickenbach et al., 1995). This program involves the transcriptional activation of specific sets of genes, which are found in the epidermal differentiation complex (EDC) of mouse chromosome 3 (Marshall et al., 2001; Martin et al., 2004). The coordinated expression of these genes eventually leads to the development of the cornified envelope, which forms a scaffold for the barrier lipids. Additionally, the tight junction barrier, which has been shown to depend on Claudin 1 and E-cadherin function (Furuse et al., 2002; Tunggal et al., 2005), is a crucial part of the granular layer. Finally, the epidermal morphogenesis is completed at E18.5 when a fully functional epidermal barrier is formed.

Several signaling pathways have been implicated in the regulation of different steps of this morphogenetic stratification program. For example, Notch signaling was shown to be essential for the onset of stratification and formation of spinous layers as loss of all Notch activity, or its processing enzyme ADAM10 in the epidermis resulted in hyperproliferation and inhibited the induction of the differentiation markers K1 and involucrin resulting in a reduced number of spinous layers (Rangarajan et al., 2001; Blanpain et al., 2006; Weber et al., 2011). Another pathway involves serine/threonine protein kinase C (PKC) activity that specifically regulates the transition from spinous

to granular layers, which was indicated by the down regulation of K10 and the induction of loricrin *in vitro* in keratinocytes (Dlugosz and Yuspa, 1993).

However, how all these signaling cascades involved in the different steps during this stratification process are orchestrated requires further investigation. To date, only the transcription factor p63 has been implicated in most of these processes, the onset of epidermal specification, regulation of ACD/SCD, control of proliferative potential, differentiation and formation of the barrier (Koster and Roop, 2007). This transcription factor is therefore regarded as the “master regulator“ of epidermal morphogenesis.

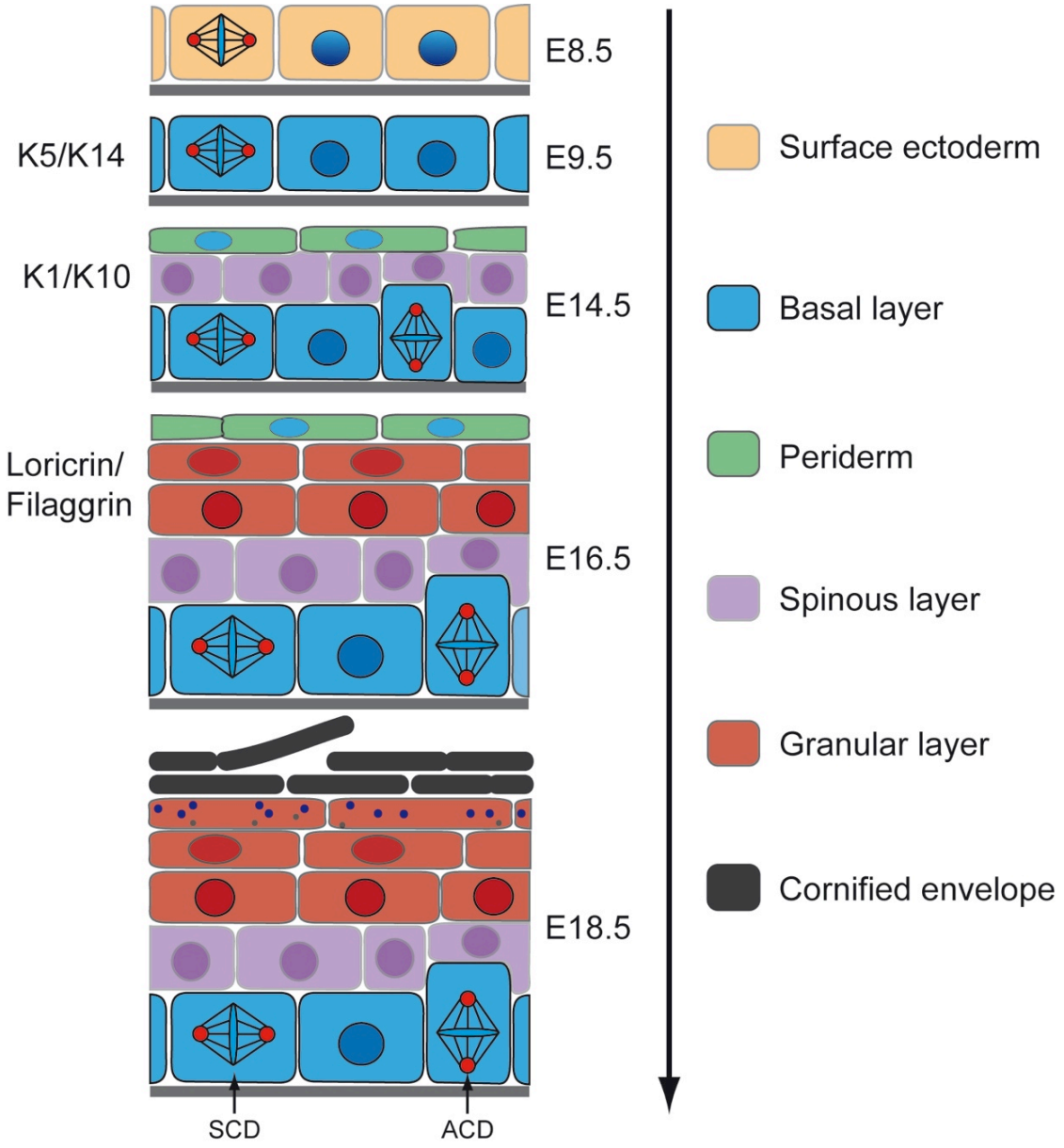


Figure 2: Schematic representation of epidermal morphogenesis. During murine epidermal morphogenesis from E8.5 to E18.5, the single layered surface ectoderm initiates a stratification program leading to the formation of the different epidermal layers with the different stratification markers (left) and eventually the barrier. SCD- symmetric cell division, ACD-asymmetric cell division

1.2.1. Asymmetric cell division in the epidermis

Asymmetric cell division (ACD) is the process by which a cell divides into two daughter cells with differential cell fates (Fig. 3). In contrast to ACDs in symmetric cell divisions (SCDs) the two daughter cells maintain the same fate. Studies in *C. elegans* and *Drosophila melanogaster* were instrumental to unravel the conserved molecular machinery regulating ACDs (Fig. 3) (Morin and Bellaiche, 2011). In Drosophila neuroblasts, polarity signaling coordinates the orientation of the spindle with the asymmetric distribution of cell fate determinants. The initial polarity of these cells is achieved by apical localization of the partitioning defect (Par)-complex, which consists of Par3, Par6 and the atypical protein kinase C (aPKC), resulting in a neuroblast cell with an apical-basal axis along which the spindle aligns (Knoblich, 2008; Poulson and Lechler, 2012). This polarity is transmitted to the spindle by the protein Inscutable (Insc), which binds directly to Par3 and forms an apical cortex (Schober et al., 1999). This alignment is accompanied by the basal localization of cell fate determinants, including Numb (Knoblich, 2010; Poulson and Lechler, 2012). By the interactions with further proteins involving LGN and NuMA the proper spindle alignment is achieved and the cell is able to divide asymmetrically into two daughter cells with different cell fate, one neuroblast and one ganglion mother cell (Poulson and Lechler, 2012).

In mammals, ACD provides a mechanism to generate distinct cell types and thereby can generate cellular diversity in a three dimensional tissue (Niessen et al., 2012). Already in 1970, divisions perpendicular to the basement membrane were observed in esophagus, a stratifying epithelium (Smart, 1970), suggesting that ACD might regulate stratification. More direct evidence came from a study by Lechler and Fuchs (2005), in which a shift in division orientation was linked to the initiation of stratification. Cells in the basal layer of the epidermis can divide in parallel to the

basement membrane resulting in two daughter cells, thus contributing to expansion of the surface area. In contrast, cells that dividing perpendicular to the basement membrane fuel the suprabasal epidermal layers. In these ACDs, one daughter remains in the basal layer and one daughter cell is committed towards differentiation (Lechler and Fuchs, 2005). At E12.5, when stratification is initiated, a major shift from SCDs to ACDs occurs in the basal layer. In these ACDs, the basal daughter expresses the basal layer marker K14 and the suprabasal daughter of the ACD begins to express K10 (Williams et al., 2011; Poulson and Lechler, 2012). Interestingly, this switch towards more asymmetrical divisions was completely lost in mice lacking p63 (Lechler and Fuchs, 2005), implicating p63 in the regulation of oriented cell divisions in the epidermis. Further studies investigating the role of the polarity proteins Insc and NuMA showed that the expression of Insc and the recruitment of NuMA to the apical cell cortex are necessary for ACDs and epidermal stratification (Poulson and Lechler, 2012). Importantly, $p63^{-/-}$ did not alter the expression or localization of both proteins, suggesting a more indirect regulation of ACD by p63 (Lechler and Fuchs, 2005). Additionally, knockdown of either LGN or NuMA during initiation of stratification resulted in a shift from ACDs to SCDs. This switch was accompanied by a reduction in Notch signaling, which is important for suprabasal cell differentiation (Williams et al., 2011). Interestingly, this depletion did not affect the Par-complex localization, suggesting that aPKC and Par3 are upstream of NuMA and LGN in the regulation of ACD (Williams et al., 2011). In line with this, the epidermal inactivation of the aPKCλ isoform produced more ACDs in the developing IFE and in different hair follicle compartments, accompanied by an altered cellular fate (Niessen et al., 2013).

These results indicate that division orientation is crucial for epidermal morphogenesis. However, whether signals from the niche control SCDs and ACDs during epidermal

morphogenesis and how p63 regulates these processes are still not known and will be important subjects for future investigations.

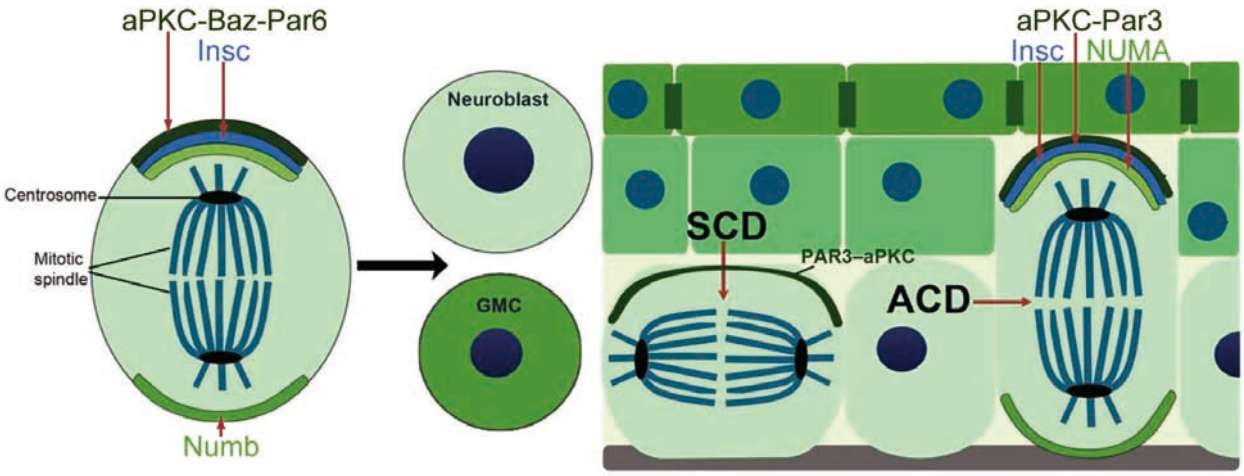


Figure 3: Asymmetric cell division in Drosophila and mammalian epidermis. In drosophila neuroblast (right panel) and SCD/ACD in the mammalian epidermis (left panel) (Niessen et al., 2012) aPKC- atypic protein kinase C, Baz- drosophila Par3, Par6- patitioning defect 6, Par3 patitioning defect 3, Insc- inscutible, NuMA- nuclear mitotic apparatus protein. Figure was adapted from Niessen et al. (2012).

1.2.2. The transcription factor p63: structure, expression and function

P63 is a member of the p53 family of transcription factors, which consists of p53, p63 and p73. All three genes share a significant sequence homology and contain a N-terminal transactivation domain, a DNA-binding domain (DBD) and an oligomerization domain (Yang et al., 2002). In addition, they all can bind DNA at a canonical p53-binding site and thereby regulate the expression of a subset of similar target genes (Yang et al., 2002; Yang et al., 2006).

P63 is a tetrameric transcription factor that is expressed from two different promoters, thereby generating at least six different isoforms with identical DBD, but different transactivation capabilities (Yang et al., 1998). Three of these isoforms contain a N-terminal transactivation domain (TA) similar to p53 whereas three Δ N isoforms

contain a shorter alternative TA domain (Yang et al., 1998) (Fig. 4). Furthermore, the p63α isoforms contain a C-terminal sterile alpha motif (SAM), thus suggesting the interaction with other co-regulating proteins (Yang et al., 1998; Thanos and Bowie, 1999; Cicero et al., 2006). Initially, it was thought that the ΔN isoforms were unable to induce target gene expression due to the presence of the alternative TA domain and therefore would function as dominant negative transcriptional repressors. However, later it was shown that ectopic ΔNp63 isoform expression could transactivate p53 reporter and induce expression of endogenous p53 targets (Dohn et al., 2001; Ghioni et al., 2002; King et al., 2003).

At present, contradictory opinions about the expression of the different p63 isoforms during surface ectoderm initiation and epidermal morphogenesis exist. One model suggests that TAp63α functions as a molecular switch to initiate epithelial stratification as it is expressed as one of the first genes in the surface ectoderm prior to the expression of K5/K14 at E7.5 (Koster et al., 2004; Romano et al., 2009). Indeed, induced ectopic expression of TAp63 in simple epithelia induced a stratification program (Koster et al., 2004). ΔNp63α, the major isoform responsible for epidermal development and homeostasis, suppresses TAp63α and is expressed later at E8.5 (Koster et al., 2004; Laurikkala et al., 2006). During ectodermal specification Bmp signaling is essential for the initiation of p63 expression, which blocks neural development and promotes epidermal commitment (Bakkers et al., 2002; Aberdam et al., 2007).

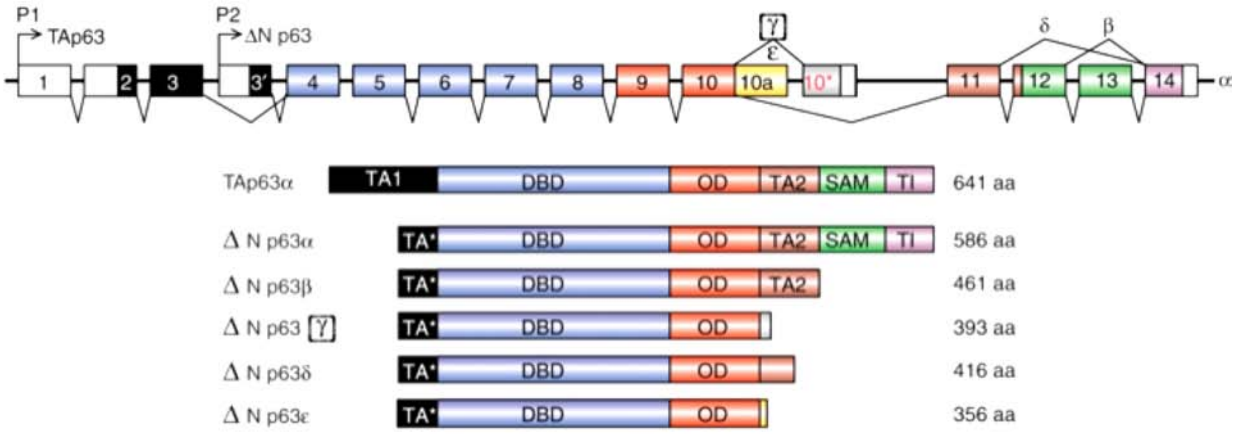


Figure 4: Schematic representation of p63 isoforms. Exon/intron organization of the human p63 gene and the alternative promoter and splicing site that give rise to the six different p63 isoforms (Vanbokhoven et al., 2011). DBD- DNA-binding domain, aa- amino acids, OD- oligomerization domain, SAM- sterile alpha motif, TA- transactivation domain, TI- transactivation inhibitory domain. (adapted from Vanbokhoven et al. 2011)

The importance of p63 for stratifying epithelia became first obvious when mice lacking p63 were investigated. These mice die from dehydration shortly after birth and display cleft palate, limb truncation and fail to develop all stratified epithelia, including the epidermis (Mills et al., 1999; Yang et al., 1999). This severe epidermal phenotype indicated a non-redundant role for p63 in this tissue and was associated with the loss of proliferative potential in progenitor cells (Yang et al., 1999; Senoo et al., 2007), impaired epidermal stratification and alterations in the differentiation of keratinocytes (Mills et al., 1999; Koster et al., 2004).

This key role for p63 as regulator of ectodermal development is in line with observations in human syndromes that have been linked to mutations in p63 resulting in altered p63 function. These ectodermal dysplasia (ED) syndromes are characterized by split hand/foot malformations and orofacial clefting. It was shown that dominant mutations in the p63 gene can cause at least five different syndromes: ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC),

ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), limb-mammary syndrome (LMS), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT) and Rapp-Hodgkin syndrome (RHS). One of the main characteristics of these syndromes is ED. In this condition skin, hair, teeth, nails and several glands are usually abnormally developed. In extreme cases large areas of the skin can be eroded. The genotype/phenotype correlations of these patients can provide information about the function of the different p63 domains in the different ectodermal compartments (Rinne et al., 2007).

For example, EEC syndrome is associated with mutations in the DBD of the p63 gene and thereby impairs the binding of p63 to the DNA (Rinne et al., 2007). One potential target that is miss-regulated by altered DNA-binding of p63 is the interferon-regulated factor 6 (IRF6) as mutations in the gene encoding this protein display similar phenotypic outcome as p63 mutations (Thomason et al., 2010; Moretti et al., 2010). These patients suffer mainly from cleft lip/palate (40% of the patients), which demonstrates the importance of this domain for craniofacial development (Rinne et al., 2007). AEC and RHS syndrome patients in contrast, mainly harbor mutations in the SAM domain of p63 (Rinne et al., 2007). The SAM domain is known to be involved in protein-protein interactions and mutations in this domain are thus most likely hampering the binding to interacting proteins (Rinne et al., 2007). The main features of AEC/RHS patients are nail and teeth defect (80% of patients), hair defects e.g. alopecia (94% of patients) and severe skin erosions (80% of patients), indicating the crucial role for p63-protein interactions in the regulation of specific target gene (Rinne et al., 2007).

In the epidermis p63 is predominantly expressed in the basal and the spinous layer and is downregulated upon initiation of differentiation in the suprabasal layers (Yang et al., 1999; Westfall et al., 2003; Koster et al., 2004; Koster and Roop, 2007). In the

basal layer p63 was shown to maintain keratinocyte proliferative potential and regulates cell adhesion (Yang et al., 1999; Truong et al., 2006; Carroll et al., 2006). It was proposed that p63 regulates proliferation by preventing senescence, as shown by the loss of the senescence markers p16 and promyelocytic leukemia (PML) protein in p63 deficient cells (Keyes et al., 2005). In addition, p63 promotes cell cycle progression by transcriptional regulation of genes, such as p21, p57Kip2 and Skp2 (Westfall et al., 2003; Beretta et al., 2005; McDade et al.). However, the cell cycle defect that was observed in p63-depleted keratinocytes could be rescued by the down-regulation of p53, indicating that p63 effects on proliferation are at least in part dependent on p53 (Truong et al., 2006). As p63 is able to regulate p53 genes (King et al., 2003; Westfall et al., 2003), it is possible that p53 and p63 compete for the same p53 consensus sequences and thereby regulate a similar set of target genes in the opposing manner (Westfall et al., 2003). Moreover, p63 knockdown in keratinocytes resulted in the down-regulation of adhesion genes, such as integrin α3 and integrin β4, and in cell detachment, suggesting a role for p63 in the regulation of cell adhesion (Carroll et al., 2006).

In addition to proliferation and adhesion, ΔNp63α regulates keratinocyte differentiation and epidermal stratification. This becomes evident by the investigation of its transcriptional targets. Thus, p63 inhibits a range of proteins that are implicated in cell cycle arrest and differentiation. For example, 14-3-3σ is suppressed by p63 in the basal layer. Suprabasal commitment of keratinocytes was accompanied by the loss of p63 repression on 14-3-3σ allowing 14-3-3σ to induce, most likely, a G2/M arrest (Hermeking et al., 1997) resulting in the cell cycle exit of these differentiating keratinocytes (Pellegrini et al., 2001). In agreement mice that overexpressed 14-3-3σ in the basal epidermal layer display a hypoplastic phenotype and fail to develop a stratified epidermis (Cianfarani et al., 2011). Another example of a p63 target gene

implicated in epidermal differentiation is Zfp750. This transcription factor promotes terminal differentiation by transactivation of another transcription factor, the Krüppel-like factor 4 (Klf4) (Sen et al., 2012), which is required for the establishment of the epidermal barrier function (Segre et al., 1999). Moreover, p63 regulates several genes of the EDC indirectly by epigenetic modulation. In mice the special AT-rich sequence-binding protein 1 (Satb1), another direct p63 target, functions as chromatin remodeler regulating the EDC locus opening during terminal differentiation of keratinocytes and thereby allows the expression of these genes, such as late cornified envelope 3c (*Lce3c*) and *Involucrin* (Fessing et al., 2011). Thus, p63 has been implicated in both early and late steps of the epidermal stratification program. Understanding how p63 orchestrates proliferation, differentiation and stratification will be essential to understand how mutations in p63 can result in such a wide spectrum of patient phenotypes and will thus require further investigation. In addition, the identification of upstream signals that regulate the different functions of p63 during morphogenesis and homeostasis is another important research area for the future.

1.3. Insulin/IGF-1 signaling

Insulin and insulin-like growth factor 1 (IGF-1) are endocrine hormones with high structural similarity. Insulin is produced by the β-cells in the pancreas and is central for the regulation of carbohydrate metabolism. IGF-1 is primarily produced by the liver, but can also be secreted by other tissues, including stromal fibroblasts. Both ligands control energy metabolism and tissue growth through binding to their receptors. The insulin-like growth factor 1 receptor (IGF-1R) and the insulin receptor (IR) are related tyrosine kinase transmembrane proteins, which are ubiquitously expressed. Thus, IGF-1 binds the IGF-1R with high and the IR with low affinity the IR and vice versa insulin binds to the IR with high and to the IGF-1R with low affinity.

Furthermore, both receptors can form heterodimers and thereby fine-tune the response to ligand binding. In addition, the insulin-like growth factor 2 (IGF-2) ligand is also able to bind, with reduced affinity, to both receptors. In contrast to the IR and IGF-1R, the insulin-like growth factor 2 receptor (IGF-2R) has no reported tyrosine kinase activity (Baserga et al., 1997; Pollak et al., 2004).

The IR and the IGF-1R share approximately 70% amino acid identity and both are synthesized as single chain pre-proreceptors. The processed proreceptors are further glycosylated, proteolytic cleaved and finally cross-linked by cysteine bonds to form a $\alpha\beta$ transmembrane protein. Whereas the putative ligand-binding site is found in the extracellular α subunit, the tyrosine kinase domain is located in the intracellular β subunit. Ligand binding to IR or IGF-1R results in kinase activation and autophosphorylation on three conserved tyrosines in the kinase domain, which further leads to phosphorylation at carboxy-terminal and juxta-membrane tyrosines. This results in the recruitment and subsequent phosphorylation of specific binding proteins, such as insulin receptor substrate 1 (IRS1) (Pollak et al., 2004). These molecules can activate different signaling cascades. For example, upon activation IRS1 can recruit and activate the phosphoinositide-3 kinase (PI3K). This interaction results in the accumulation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn activates the phosphoinositide dependent kinase 1 (PDK1) resulting finally in the activation of protein kinase B (PKB also known as Akt). Akt itself can regulate a plethora of downstream signaling targets, such as Forkhead box O transcription factors (Fig. 5) (Ogg et al., 1997). Additional downstream effectors of IR/IGF-1R (IIS) include the mammalian target of rapamycin complex (mTORC), extracellular signal-regulated kinases (ERK) and c-jun N-terminal kinases (JNK) (Pollak et al., 2004).

Although structurally very similar, the function of IR and IGF-1R can be quite distinct in different tissues. Thus, the IR was suggested to be more important for energy

metabolism, as demonstrated by $IR^{-/-}$ mice, which are born largely normal, but have an early onset of diabetes and die due to ketoacidosis (Accili et al., 1996; Pollak et al., 2004). $IGF\text{-}1R^{-/-}$ mice, in contrast, are much smaller then control mice at birth (±45% of normal size) and die due to severe organ hypoplasia, thus indicating the importance of IGF-1 for tissue growth (Liu et al., 1993). Nevertheless, IR/IGF-1R double knockout studies indicate an additional growth related role of the IR during embryogenesis (Kitamura et al., 2003). Single mutations ablating IR function result in embryos that are 90% of normal size, whereas single IGF-1R mutations result in small embryos (±45% of normal size). Combined ablation of IR and IGF-1R, however, resulted in even smaller embryos that are 30% of normal size, demonstrating a growth-promoting role during embryogenesis not only for the IGF-1R but also for IR (Kitamura et al., 2003).

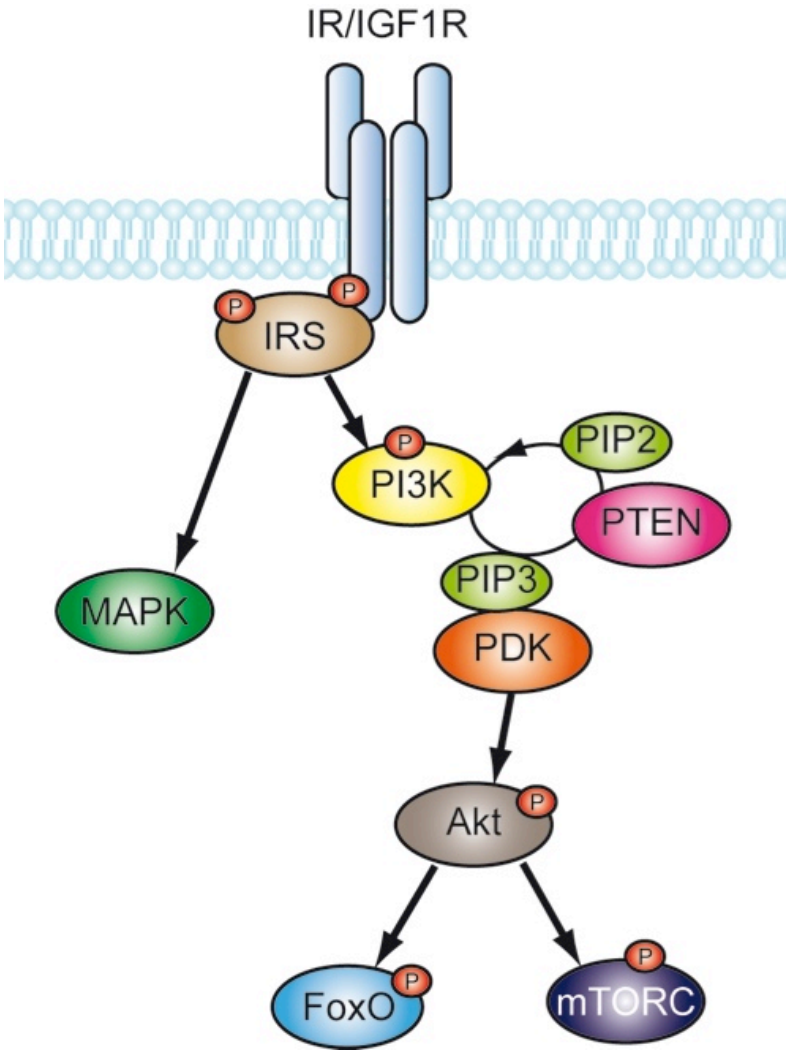


Figure 5: Schematic representation of the IR/IGF-1R signaling pathway. The insulin/IGF-1 binding dependent signaling cascade with conserved downstream regulators. IRS- insulin receptor substrate, PI3K- phosphoinositide-3 kinase, PIP3- phosphatidylinositol 3,4,5-trisphosphate, PDK- phosphoinositide dependent kinase, PTEN- phosphatase and tensin homolog, Akt- protein kinase B, FoxO- forkhead box O factor, mTORC- mammalian target of rapamycin complex, MAPK- mitogen activated protein kinase

1.3.1. FoxO transcription factors

Forkhead box O transcription factors (FoxOs) belong to the family of FOX transcription factors. The defining feature of this family is a 80-100 amino acid motif that binds DNA. This motif is a highly conserved “winged-helix” DBD and was first described in *Drosophila melanogaster* (Lam et al., 2013). The sub-family of FoxO proteins mainly acts as transcriptional activators by binding to a conserved TTGTTTAC consensus core recognition motif in diverse target genes (Furuyama et al., 2000) thereby controlling various cellular processes including cell cycle, cell survival and metabolism by regulating the expression of diverse target genes (Calnan and Brunet, 2008). FoxOs are highly conserved and in mammals consists of four members, FoxO1, FoxO3, FoxO4 and FoxO6. They are controlled by a wide range of stimuli, such as growth factors, nutrients, cytokines and oxidative stress stimuli (Fig. 6) (Calnan and Brunet, 2008; Eijkelenboom and Burgering, 2013). For example, insulin/IGF-1, act through PI3K to activate PDK1 and subsequently Akt. Active Akt then translocates to the nucleus and phosphorylates FoxO at three conserved phosphorylation sites leading to nuclear export and cytoplasmic retention of FoxO (Fig. 7) (Calnan and Brunet, 2008; Eijkelenboom and Burgering, 2013). FoxO6 in contrast lacks the third phosphorylation site and is not regulated by nucleo-cytoplasmic shuttling (Jacobs et al., 2003). In addition, stress signaling (mainly high levels of reactive oxygen species) activates FoxO in the opposite way and induces translocation to the nucleus. This was initially shown to involve JNK-mediated phosphorylation of FoxO (Eijkelenboom and Burgering, 2013).

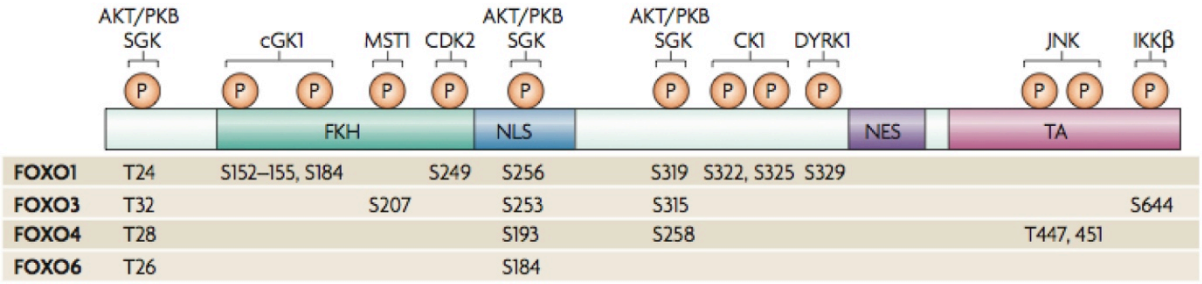


Figure 6: Schematic representation of human FoxO isoforms. The structure and the phosphorylation sites of the different mammalian FoxO isoforms are shown. FKH- forkhead domain, NLS- nuclear localization signal, NES- nuclear export sequence, TA- transactivation domain. (adapted from van der Horst et al. 2007)

The FoxO homologue Daf-16 in *C. elegans* extends longevity (Lin et al., 1997; Ogg et al., 1997; van der Horst and Burgering, 2007). The effects of loss of the *C. elegans* homologue Daf-2 resulting in longevity, were bypassed by the loss of Daf-16, indicating that Daf-2 antagonizes Daf-16 function. In *C. elegans*, loss of Daf-2 leads to a developmentally arrested dauer stage, but animals bearing weak Daf-2 mutants could develop reproductively, but showed increased energy storage and longevity (Lin et al., 1997; Ogg et al., 1997). These findings suggested a potential role for the insulin/IGF-1/FoxO axis in longevity and ageing of higher organisms. In agreement, deregulation of FoxO activity was identified in several age-related diseases, such as cancer and diabetes (van der Horst and Burgering, 2007; Eijkelenboom and Burgering, 2013).

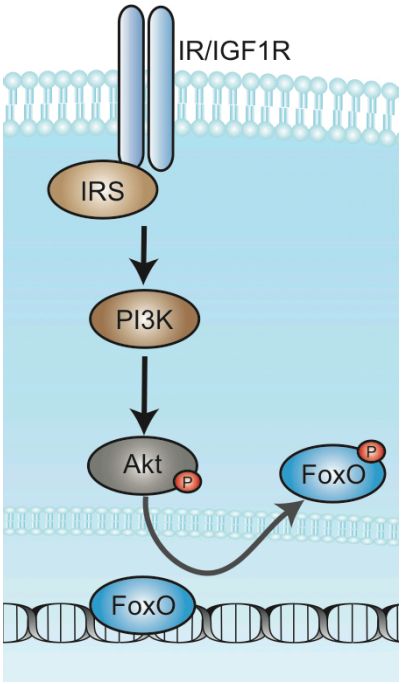


Figure 7: Simplifies Scheme of insulin/IGF-1 mediated regulation of FoxOs. Insulin/IGF-1 regulate FoxO cytoplasmic retention by Akt dependent phosphorylation. Upon ligand binding IR/IGF-1R phosphorylate Akt via IRS and PI3K. Akt translocates to the nucleus and phosphorylates FoxOs. Phosphorylated FoxOs are actively exported from the nucleus. IRS- insulin receptor substrate, PI3K- phosphoinositide-3 kinase, Akt- protein kinase B, FoxO- forkhead box O factor

1.3.2. Regulation of FoxO transcriptional activity

As shown by the IIS/PI3K/Akt mediated cytoplasmic retention, FoxO regulation is achieved mainly by post-translational modification (PTM). Phosphorylation of FoxO at the first and the second phosphorylation site that regulate nuclear export creates binding sites for the chaperone protein 14-3-3 (Brunet et al., 1999; Obsilova et al., 2005). This binding allows the active export of FoxOs by exposing the nuclear export sequence (Brunet et al., 2002). Additionally, the second phosphorylation site prevents re-entry to the nucleus by the introduction of a positive charge in the nuclear localization signal (Rena et al., 2001). Multiple other kinases can also regulate FoxO

activity at other sites than those targeted by Akt and regulate FoxO cytoplasmic sequestration (Rena et al., 2001). E.g. cyclin dependent kinase 1 and 2 are able to phosphorylate FoxO1 at serine 249 and also induce sequestration in the cytoplasm (Huang et al., 2006; Yuan et al., 2008). Although this is connected to DNA-damage responses the functional consequence of this is unclear.

Vice versa stress stimuli promote nuclear translocation of FoxOs, even in the presence of growth factors. In response to oxidative stress, the kinase MST1 phosphorylates FoxO3 at Ser207 (human) in the DNA-binding domain and disrupts 14-3-3 binding. This enables FoxO3 re-localization from the cytoplasm to the nucleus (Lehtinen et al., 2006; Calnan and Brunet, 2008). Similarly, the stress activated protein kinase JNK phosphorylates FoxO4 at two phosphorylation sites (Thr447 and Thr 451), which also results in FoxO nuclear localization (Essers et al., 2004).

The AMP-activated protein kinase (AMPK), which has a central role in cellular energy homeostasis controls cell growth and energy expenditure by among others phosphorylating FoxO3 at three conserved serine residues (Ser413, Ser588 and Ser626) (Hardie et al., 2012). This leads to FoxO transcriptional activity in response to low energy levels and contributes to stress resistance, by the up-regulation of the superoxide dismutase Sod3 (Greer et al., 2007). This activation of FoxO is independent of the regulation of its subcellular localization and also independent of additional growth factor stimulation.

Acetylation is another PTM that can regulate FoxO nuclear activity. For example, Histone deacytelases, including sirtuins (Sirt), regulate FoxO acetylation leading to changes in FoxO DNA-binding capacities in response to stress signaling (Brunet et al., 2004; van der Horst et al., 2004). Thus, the de-acetylation of FoxO by Sirt1 has been proposed to induce a switch in gene expression from apoptosis to stress resistance and cell cycle arrest. This was shown by the deletion of Sirt in mouse

embryonic fibroblasts (MEFs), which reduced FoxO-dependent CDKN1B and GADD45 expression but not the pro apoptotic gene BCL2 (Brunet et al., 2004).

In response to insulin and growth factor signaling through the PI3K-Akt pathway, FoxOs are not only sequestered in the cytoplasm but also can be targeted for degradation. This degradation is also induced by Akt phosphorylation resulting in poly-ubiquitinylation of FoxO by the E3 ligase SCFskp2 complex (Huang et al., 2005). Moreover, the interaction of FoxOs with other transcription factors creates further variation in the transcriptional output in different cell types. Through these interactions FoxOs are able to mediate crosstalk with other signaling pathways and integrate their upstream information into target gene regulation. Several transcriptional interaction partners have been described. FoxOs can bind nuclear receptors (e.g. oestrogen receptor, retinoic acid receptors), metabolic regulators (PPARγ) and transcription factors (e.g. SMAD proteins) (Eijkelenboom and Burgering, 2013).

Interestingly, FoxO and p53 are both involved in regulation of cell cycle progression and cellular survival. They also both can control expression of enzymes such as Sirt1 and MDM2. Moreover, it was shown that in response to nutrient deprivation FoxO releases p53 mediated Sirt1 repression and thereby leads to Sirt1 up regulation. This repression appeared to be independent of the presence of a FoxO-binding site in the Sirt1 promoter (Nemoto et al., 2004). This binding is mediated by two conserved regions in the FH domain and the C-terminal transactivation domain of FoxO, which interact with the DNA-binding domain of p53 (Wang et al., 2008). Thus, FoxO may also regulate gene expression independent of FoxO binding to DNA.

Further, it was proposed that FoxO and the myelocytomatosis viral oncogene (MYC) act as antagonists during the regulation of mitochondrial homeostasis. Hypoxia induced binding of FoxO3 to the promoter of mitochondrial genes is inversely

correlated with MYC promoter binding. Thus, siRNA knockdown of FoxO3 increased the binding of MYC to these promoters suggesting that FoxO3 directly inhibits MYC DNA interactions. Generally, FoxO and MYC directly and indirectly inhibit each other and have opposing effects on proliferation and energy consumption independent of FoxO-DNA binding (Jensen et al., 2011).

It was also demonstrated that upon increased oxidative stress FoxO and β-catenin can interact and regulate FoxO transcriptional activity. β-catenin is a transcriptional co-activator downstream of Wnt signaling that binds to and activates TCF (T cell factor) transcription factors. Interestingly, in osteoblasts oxidative stress causes a switch from TCF dependent transcription to FoxO-dependent target expression. This is most likely due to FoxO-sequestration of β-catenin away from TCF (Essers et al., 2005; Hoogeboom et al., 2008). Overall, FoxO transcription factors function at the intersection of numerous signaling pathways and transmit external stimuli into transcriptional regulation of the appropriate target genes. Therefore, FoxO function has to be fine-tuned at various levels to induce the proper cellular responses.

1.3.3. FoxO function in stem cells

FoxO transcription factors mainly function to regulate cellular and organismal homeostasis. In addition to their roles in differentiated cells they also are critical to limit the expansion of stem and progenitor cells of tissues.

In the hematopoietic system FoxOs normally limit the expansion of myeloid and lymphoid cell lineages by inhibiting stem cell proliferation and maintaining quiescence (Eijkelenboom and Burgering, 2013). Conditional ablation of FoxO1, -3 and -4 in hematopoietic system resulted in hematopoietic stem cell (HSC) pool exhaustion and increased reactive oxygen species (ROS) and apoptosis. HSCs are considered to be quiescent stem cells and FoxOs control the G0 to G1 and G1 to S phase progression

during cell cycle through the regulation of transcriptional targets including *CCNG2*, *CDKN1A*, *CDKN1B* and *CCND2*. In addition, they protect HSCs from cell death and maintain their self-renewal capacity, which is mediated by the resistance to oxidative stress through upregulation of Sod2, Catalase, ATM and CDKN2A (p16) (Tothova et al., 2007; Miyamoto et al., 2007).

In neural stem cells (NSC), FoxO family members play a prominent role in proliferation and stem cell renewal (Eijkelenboom and Burgering, 2013). Combined knockout of FoxO1, 3 and 4 in the brain of mice resulted in increased brain size and proliferation of neural progenitor cells during early postnatal life. In adults, the effect was reversed and resulted in a decline of NSC pool. Similar to HSCs, FoxOs regulates genes and pathways that control cellular proliferation, differentiation and oxidative defense by the up-regulation of several cyclins and cyclin dependent kinases (CDKs) and the suppression of p57/KIP2 (Paik et al., 2009).

Together, the data on FoxO function in HSCs and NSCs suggest a general mechanism by which FoxOs mediate self-renewal and stem cell maintenance of adult stem cells through transcriptional regulation of cell cycle arrest and oxidative stress resistance.

1.3.4. FoxO function in the epidermis

The role of FoxOs in epidermal keratinocyte homeostasis is largely unknown. However, signals crucial for epidermal morphogenesis, such as tumor necrosis factor α (TNFα) or Notch can regulate FoxO activity in other tissues (Hu et al., 2004; Hu and Hung, 2005; Kitamura et al., 2007). In human keratinocytes FoxOs were implicated in regulating immediate gene activation responses to tumor growth factor-β1 (TGF-β1) treatment (Gomis et al., 2006). Interestingly, activation of these target genes required Smad4 as a co-regulator of FoxOs. Among the FoxO-Smad regulated

genes were stress response genes, including *GADD45A* and *GADD45B*, and adaptive cell signaling response genes, such as *JAG1*, *OVOL1* and *CTGF* (Gomis et al., 2006). Furthermore, in murine wound healing experiments FoxO1 promoted keratinocyte migration and blocked apoptosis, which is in contrast to other cell types where it inhibits proliferation and induced apoptosis (Eijkelenboom and Burgering, 2013; Ponugoti et al., 2013).

These data thus indicate that FoxOs may integrate multiple upstream signals to differentially regulate proliferation and oxidative stress responses also during epidermal development and homeostasis.

1.3.5. IIS/FoxO in skin pathogenesis

Several insulin/IGF-1 related skin conditions have been described, such as impaired wound healing in type 2 diabetes mellitus, psoriasis and skin cancer, that correlate with and might be due to an altered insulin/IGF-1 function.

Diabetes affects 8.3% (2013) of the population worldwide and more then 80% of that account for type 2 diabetes mellitus (Tamayo et al., 2013). Type 2 diabetes is a result of impaired insulin action and inadequate insulin secretion from the β-cells of the pancreas. Dermatologic problems are common in type 2 diabetes patients and often involve bacterial skin infections and skin barrier breakdown. These conditions appear to be secondary to multiple factors, including poor microvasculature, peripheral neuropathy and the decreased immune response (Ahmed and Goldstein, 2006). Some of these factors may also contribute to impaired wound healing, which is the major characteristic of diabetic skin complications with an incidence of about 15% of all diabetic patients (Faglia et al., 2001). These wound closure problems can lead to severe ulcerations, diabetic foot and ultimately to amputations. However, the role of IR/IGF-1R signaling in diabetes-associated wound healing problems in the skin is not

clear. In a well-characterized mouse model of obese mice (Ob/Ob mice) that ultimately develop diabetes severe impairment of insulin signaling in the skin was observed in wound healing experiments (Goren et al., 2006). Accordingly, conditional overexpression of the insulin relative IGF-1 in murine keratinocytes accelerated wound closure (Semenova et al., 2008), suggesting the involvement of skin IIS in these processes.

Insulin resistance in diabetes can compromise negative regulation of FoxOs. This has been observed in various tissues and cell types in connection with diabetes, such as liver, pancreas and adipose tissue (Nakae et al., 2008). However, the role of FoxOs in diabetic skin complications is unknown. Interestingly, conditional inactivation of FoxO1 in murine epidermis altered wound closure, suggesting a promoting role for FoxO in tissue regeneration. Surprisingly, FoxO regulated keratinocyte migration by up-regulation of TGF-β1 signaling (Ponugoti et al., 2013).

Psoriasis, a common, chronic skin disease that affects approximately 2% of the population is another skin disease associated with impaired insulin function (Christophers, 2001). Around 85-90% of these patients suffer from the most common variant psoriasis vulgaris (Griffiths and Barker, 2007). One main feature of this disease are scales, which are the result of a hyper-proliferative epidermis with premature keratinocytes and incomplete cornification with retention of nuclei in the *stratum corneum* (Nestle et al., 2009) resulting in skin barrier impairment. This in combination with dermal inflammatory infiltrate, which consists mainly of dendritic cells, macrophages and neutrophils, contributes to the overall thickness of psoriatic lesions (Nestle et al., 2009). Population based studies have shown that psoriasis is linked to diabetes mellitus and insulin resistance (Takahashi and Iizuka, 2012). How both conditions are linked is still not completely understood. One recent study suggested the involvement of inflammatory cytokine interleukin 1β (IL-1β). IL-1β is

present in high quantities in psoriatic lesions and was suggested to induce insulin resistance through p38MAPK (mitogen-activated protein kinase) signaling resulting in a block of insulin-dependent differentiation of keratinocytes (Buerger et al., 2012). In addition, IGF-1, secreted by dermal fibroblasts, may contribute to the epidermal hyperplasia of psoriasis by promoting keratinocyte proliferation (Krane et al., 1992; Miura et al., 2000). In line with this, targeting the IGF-1R by oligonucleotide interference reduced epidermal hyperproliferation in psoriasis (Wraight et al., 2000).

Additionally, several downstream signaling targets of insulin/IGF-1 have been studied in the context of psoriasis. The PI3K/Akt axis e.g. was proposed to activate the anti-apoptotic nuclear factor-κ B signaling cascade and inhibits pro-apoptotic Bcl2-associated death promoter (BAD) signaling in keratinocytes, which contributes to the thickening of psoriatic skin (Madonna et al., 2012). Although the functional role for FoxOs in psoriasis is not known, pathways that have been demonstrated to regulate FoxO activity are essential for psoriasis pathogenesis, such as TNF receptor signaling, suggesting potential involvement of FoxOs (Hu and Hung, 2005; Hu et al., 2004; Kitamura et al., 2007; Kumari et al., 2013).

Moreover, insulin/IGF-1 and FoxO have been shown to be crucial regulators of carcinogenesis and tumor growth (Pollak, 2008; Kloet and Burgering, 2011), which indicate also a role in epidermal cancers, such as basal cell carcinoma and squamous cell carcinoma. Overall this pathway might thus be a promising candidate for potential therapies of skin diseases, such as diabetic wound problems, psoriasis and skin cancer, even though the precise role of IIS regulated FoxOs in these conditions is not fully understood.

1.3.6. IIS regulates epidermal thickness and proliferative potential

In addition to circulating insulin and IGF-1, dermal fibroblasts are the major source of IGF-1 in the skin. Epidermal keratinocytes express both receptors, the IR and the IGF-1R, but they do not produce insulin or IGF-1 ligands (Misra et al., 1986; Verrando and Ortonne, 1984; Wertheimer et al., 2000). In the skin, dermal fibroblasts secret IGF-1 and insulin and thereby could support keratinocyte proliferation. Interestingly, IGF-1R$^{-/-}$ mice had an abnormal thin skin with translucent epidermis, indicating a role for IGF-1 signaling in epidermal development (Liu et al., 1993). Consistently, the combined deletion of Akt1 and Akt2, the main downstream targets of the IIS pathway, also resulted in a hypoplastic skin phenotype (Peng et al., 2003). In contrast, IR$^{-/-}$ mice showed no obvious skin phenotype, suggesting that IR signaling does not directly contribute to epidermal morphogenesis and homeostasis. In summary, these data suggested a putative role for IIS in skin development primarily through IGF-1R action.

To test whether cell autonomous IIS regulates epidermal morphogenesis, conditional epidermal IR$^{epi-/-}$ and IGF-1R$^{epi-/-}$ mice were generated by K14-Cre driven inactivation (Stachelscheid et al., 2008). Investigation of these mice revealed that IR$^{epi-/-}$ mice were viable, showed no obvious macroscopic phenotype and developed normally to adulthood. In contrast, the loss of IGF-1R resulted in newborn mice with thin translucent skin and reduced survival rate (60% viable). In addition, the surviving IGF-1R$^{epi-/-}$ mice maintained a thinner epidermis throughout lifetime and showed occasional hair loss. Most interestingly, the simultaneous deletion of IR and IGF-1R (dko^{epi}) resulted in an even more severe phenotype and perinatal lethality due to a major barrier defect resulting in dehydration (Stachelscheid et al., 2008).

Histological investigation of these three different knockout mice revealed that although they have a normal macroscopic appearance, there was a slightly

decreased epidermal thickness in the IR$^{epi-/-}$ mice compared to control. This reduction in suprabasal layers was much more severe in the IGF-1R$^{epi-/-}$ mice and most severe in the dko^{epi} mice (Fig. 8A) (Stachelscheid et al., 2008). These results indicate an essential, non-redundant role for IIS during epidermal morphogenesis and furthermore show that IGF-1R is the major regulator of this signaling pathway in the epidermis.

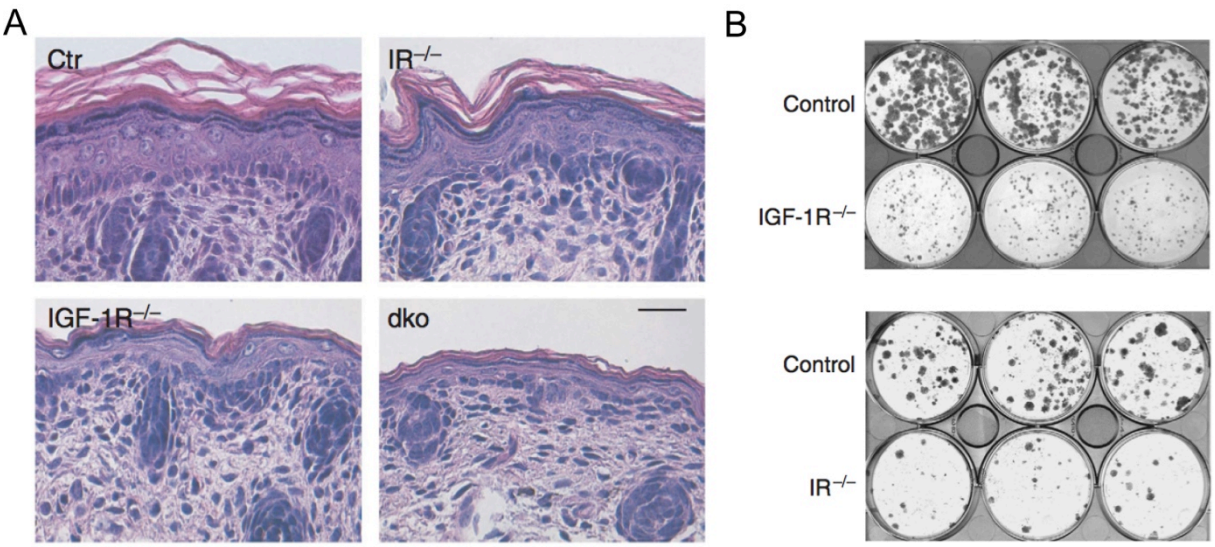


Figure 8: IIS regulates epidermal thickness and proliferative potential. (A) H&E staining of ctr, IR$^{epi-/-}$, IGF-1R$^{epi-/-}$ and dko^{epi} newborn mice show decreased epidermal thickness. (B) CFA with primary murine IR$^{epi-/-}$ and IGF-1R$^{epi-/-}$ keratinocytes indicate loss of proliferative potential in upon loss of IIS (adapted from Stachelscheid et al., 2008).

Interestingly, overall differentiation of the keratinocytes in the IGF-1R$^{epi-/-}$ and the dko^{epi} mice did not seem to be obviously impaired as the localization of markers for the different layers K14, K10 and loricrin was not altered (Stachelscheid et al., 2008). As IIS can promote cell survival through Akt activation, increased cell death could explain the loss of keratinocytes in the epidermis. However, apoptosis was not changed upon loss of IIS. Ki67 staining also revealed no obvious change in proliferation of newborn IGF-1R$^{epi-/-}$ and dko^{epi} epidermis compared to control

epidermis. This was remarkable as keratinocytes isolated from IGF-1R$^{epi-/-}$ mice were growth impaired and displayed a severe loss of proliferative potential in cell culture conditions, as assessed by colony-forming assays (CFA) (Fig. 8B). In CFAs colony-size signifies the number of cell division each cell can undergo (Izumi et al., 2007). The largest colonies are thought to represent stem cell like progenitor cells with high proliferative potential (Izumi et al., 2007). This suggested that in the epidermis the reduction of suprabasal cells was indirectly caused by a decrease in progenitor cells. In line, progenitor markers, such as Keratin 15 (K15) and IGF-binding protein 5 (Igfbp5), were down regulated upon loss of IIS *in vivo* (Stachelscheid et al., 2008). The hypomorphic epidermis and the loss of the proliferative potential were partially rescued by expression of constitutive active Rac1. Thus, IIS to regulates proliferative potential and epidermal morphogenesis through the small GTPase Rac (Stachelscheid et al., 2008).

The question remained how IIS promotes proliferative potential and how an altered proliferative potential resulted in a loss of suprabasal layers. Further analysis of different embryonic stages of these mice showed that the hypoplastic epidermis was first obvious at E16.5 (Fig. 9A), which is the developmental stage when there is a strong increase in number of suprabasal layers. Surprisingly, Ki67 labeling did not reveal any changes in proliferation at this stage, similar to what was observed in newborn mice. However, one day later at E17.5 Ki67 staining was reduced. As Ki67 is expressed in all stages of the cell cycle, it may not detect either a G2/M or mitotic arrest at E16.5. Quantification of the number of anaphase spindles at E16.5 revealed a decrease of cells in anaphase at E16.5 in the epidermis (Fig. 9B). Interestingly, this decrease was in agreement with the severity of the phenotype with dko^{epi} mice having the strongest reduction in anaphase divisions. Further analysis showed an increase of cells in metaphase spindles in E16.5 dko^{epi} epidermis and IGF-1R$^{epi-/-}$

keratinocytes concomitant with a decrease in anaphase stage, thus indicating a mitotic checkpoint arrest (Fig. 9C). This was associated with altered expression of regulators of cell cycle progression in keratinocytes, such as Mdm2, 14-3-3σ, Ccng1 and p63 in the epidermis of newborn dko^{epi} mice.

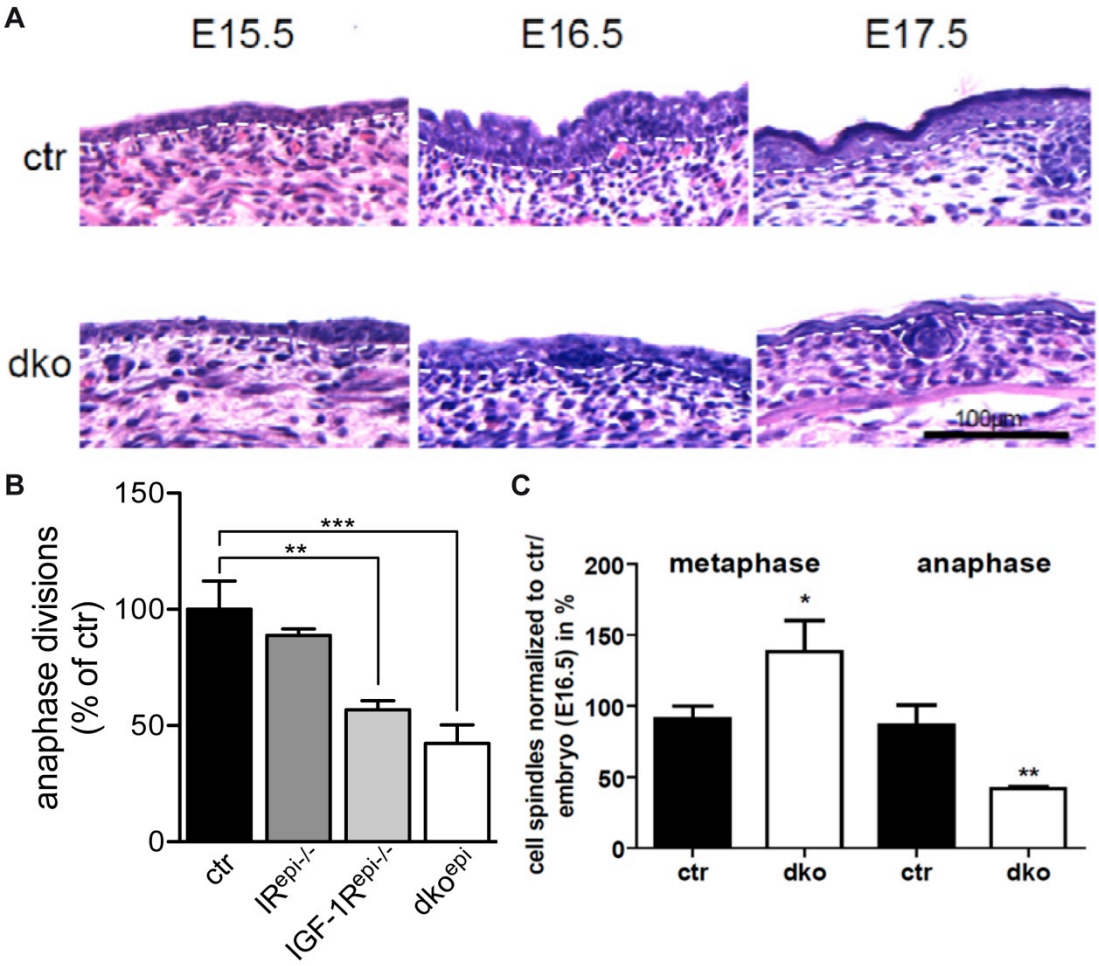


Figure 9: IIS regulates cell cycle progression at E16.5. (A) H&E staining of embryonic ctr and dko^{epi} epidermis show first appearance of hypomorphic epidermis at E16.5. (B) Less anaphase cell division upon loss of IR, IGF-1R or both (dko^{epi}) in E16.5 epidermis. (C) Quantification of metaphase and anaphase divisions in E16.5 dko^{epi} epidermis reveal a mitotic block (adapted from the PhD thesis of Heike Stachelscheid 2010).

As expansion of suprabasal layers at least in part is driven by ACD (Lechler and Fuchs, 2005; Williams et al., 2011; Poulson and Lechler, 2012) cell division orientation was quantified based on DAPI stainings. This initial analysis revealed a biased loss of ACDs in the different IIS mice, the severity of which correlated with the phenotype, thus providing a potential explanation for the reduction in suprabasal layers. These findings by Heike Stachelscheid were first indications for a temporary arrest in mitosis and a biased loss of ACDs during epidermal morphogenesis in $IR^{epi-/-}$, $IGF\text{-}1R^{epi-/-}$ and dko^{epi} mice. These data form the basis for the major aims of this thesis. They also indicate that IIS couples proliferative potential to the regulation of ACD thereby promoting the expansion of suprabasal layers and thus stratification.

1.4. Aims of this thesis

The overall aim of this thesis was to determine how IIS regulates epidermal morphogenesis and to ask whether the FoxO family of transcription factors control embryonic stratification and barrier formation in the epidermis downstream of IIS.

Specifically the following questions were addressed:

1. Does IIS control asymmetric division and mitotic progression?
2. Which FoxO transcription factors are expressed in the epidermis?
3. Is epidermal FoxO nuclear activity regulated by the loss of IIS?
4. Do FoxOs contribute to epidermal morphogenesis?
5. What are the molecular mechanisms by which the IIS/FoxO axis regulates epidermal morphogenesis?
6. Which potential transcriptional target genes are controlled by IIS/FoxO?

2. Results

2.1. Epidermal IIS controls mitotic progression

The previous findings that keratinocytes lacking the IGF-1R were arrested in the spindle checkpoint indicate that mitotic progression was regulated by IIS. Thus, we asked whether IIS is activated and necessary during mitosis. Therefore we first synchronized human HaCat keratinocytes in G1 by thymidine treatment, after G release, cells were subsequently serum starved during G2 and arrested in mitosis by treating them with nocodazole. Subsequently, these cells were allowed to progress in mitosis by removing nocodazole either in the absence of growth factors or in the presence of insulin/IGF-1 or, as a positive control, fetal calf serum (FCS). Both insulin/IGF-1 and FCS induced Akt phosphorylation, whereas no increased in phosphorylation was observed in the no FCS condition, thus indicating that IIS is activated during mitosis (Fig. 10A). More importantly, FACS analysis revealed that the addition of insulin/IGF-1 alone was sufficient to release cells from the mitotic arrest resulting in significant more cells in G1 cell cycle phase compared to serum starved cells. This indicates that IIS activity is directly required during mitosis (Fig. 10B).

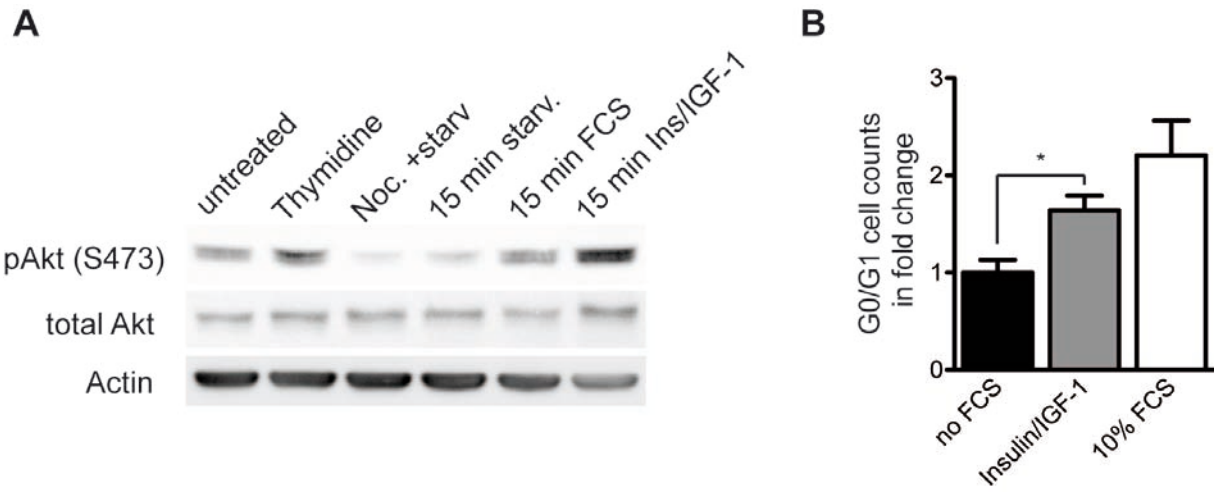


Figure 10: IIS is sufficient to drive more cells into G1-phase after cell cycle block. (A) Western blot analysis of HaCat keratinocytes, which were synchronized using thymidine, nocodazole (Noc) in combination with or without serum starvation to arrest cells in mitosis. Subsequently, cells were incubated for 15 min in starvation medium alone or containing either 10% FCS as a positive control or insulin/IGF-1, after which activation of Akt was assessed using phosphorylation of Akt Serine 473 (S473) as a read out for IIS activity. (B) Cell cycle analysis of HaCat keratinocytes, which were starved in G2 by serum removal and subsequently arrested in mitosis, by nocodazole treatment. This arrest was released by removing nocodazole either without serum or by insulin/IGF-1 alone or by FCS as positive control. Each column represents the mean of n=5 independent experiments ± SEM. Significance was tested using student' T-test and were indicated by *p< 0.05. Each column indicates the mean of n=5 independent experiments ± SEM.

2.2. Epidermal IIS promotes ACD

During embryonic development, the initiation of stratification in epidermal morphogenesis coincides with a reorientation of the mitotic spindle from parallel (SCD) to predominantly perpendicular (ACD) to the basement membrane (Lechler and Fuchs, 2005). ACD results in one basal daughter and one suprabasal daughter that differentiates (Poulson and Lechler, 2010; Williams et al., 2011), suggesting that these perpendicular divisions promote differentiation and formation of suprabasal layers. Mice lacking the epidermal IGF-1R and the IGF-1R in combination with the IR showed a severe stratification defect (Fig. 8A), which was first obvious at E16.5 (Fig.

9A). This defect was accompanied by a loss of cells in anaphase, which was most strong in IGF-1$^{epi-/-}$ and dko^{epi} epidermis (Fig. 9B). We therefore asked whether the loss of IIS in the epidermis resulted in loss of SCD and/or ACD in the basal epidermal layer. As IGF-1R$^{epi-/-}$and dko^{epi} epidermis showed a more severe reduction in suprabasal layers, skin sections of these E16.5 mice were analyzed. We used survivin staining, which marks the mid-body of anaphase and telophase spindles by two dots. The trans-section through both dots determined the axis of spindle orientation and thus of division, which was used to measure the angle to the basement membrane, marked by collagen IV (Fig. 11A). This angle measurement allowed a more precise evaluation of ACDs and SCDs compared the identification of mitotic cells by DAPI-DNA staining. The obtained angles were plotted in a radial histogram (Fig. 11B) and further categorized in SCD 0-30°, random 30-60° and ACD 60-90° (Fig. 11C). In control epidermis the majority of cells divide in a perpendicular orientation to the basement membrane with an angle of 80-90° degree, whereas less than 10% of the cells divided symmetrically with an angle of 0-10% to the basement membrane. In both IGF-1R$^{epi-/-}$ and dko^{epi} epidermis a shift from cells dividing perpendicular to cells dividing in parallel to the basement membrane was observed when compared to control E16.5 embryos (Fig. 11B). To examine whether this shift in the ratio towards SCD was due to a more extensive loss of ACD, the relative percentage of each division was plotted against the control. This revealed a biased reduction in ACD in both IGF-1R$^{epi-/-}$ (50% reduction) and dko^{epi} (85% reduction) compared to control embryos (Fig. 11C), whereas SCDs were reduced only in the dko^{epi} mice albeit to a lesser extent. These results are in agreement with the more severe phenotype of the dko^{epi} and the most extensive reduction of cells in anaphase in these mice (Fig. 9B).

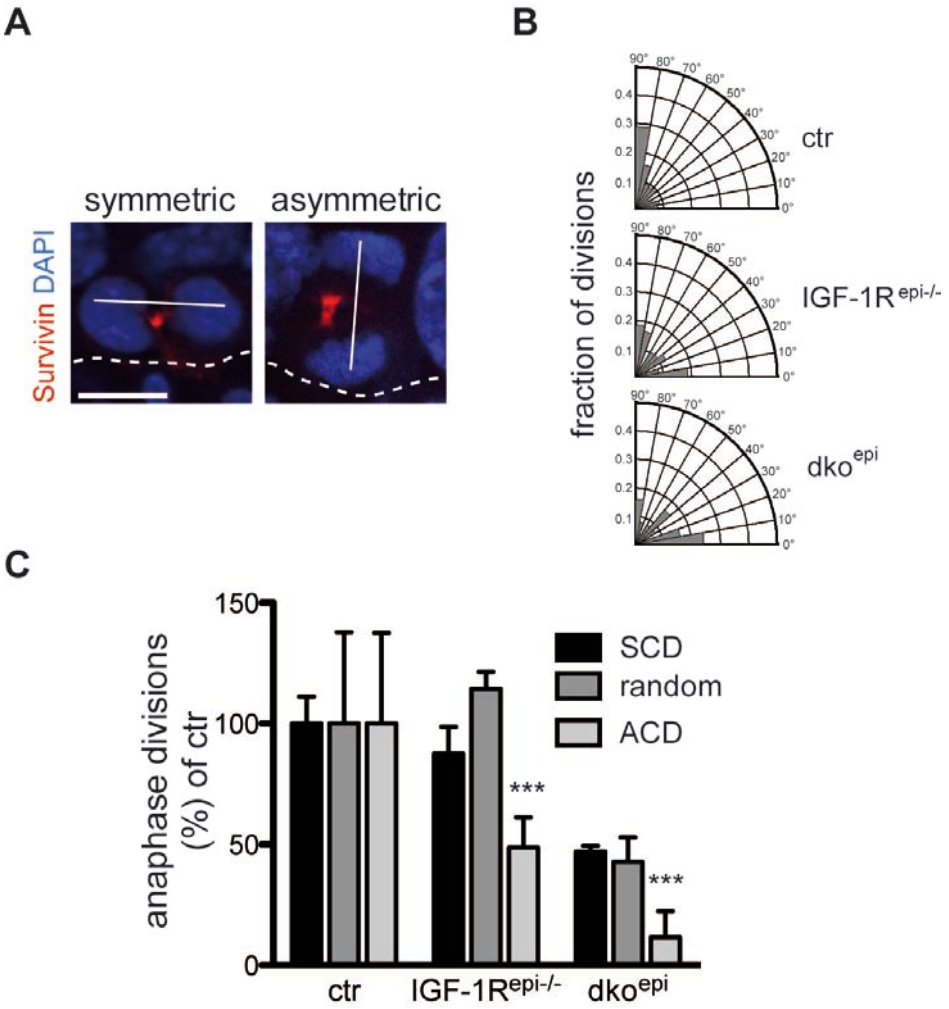


Figure 11: IIS controls epidermal stratification through regulation of asymmetric cell division. (A) Survivin staining (red) of SCD (symmetric) and ACD (asymmetric) in the basal layer of the epidermis. The dotted line indicates the basement membrane and the continuous line indicates the orientation of the division. Nuclei were counterstained with DAPI and the scale bar is 10 µm. (B) Radial histogram quantification of division angles (n=3 E16.5 embryos per genotype) shown as fraction of total division. (C) Relative comparison of different division orientations in basal keratinocytes of IGF-1R$^{epi-/-}$ (n=49 divisions), dko^{epi} (n=25 divisions) and control (n=71 divisions) categorized in 0-30° as SCD, 30-60° as random and 60-90° as ACD. Significance was tested separately for each type of division using one-way ANOVA and were indicated by *p< 0.05, **p<0.01 and ***p<0.001. Each column indicates the mean of n=3 E16.5 embryos/genotype ± SEM.

2.3. IIS regulates p63 activity in keratinocytes *in vitro*

The transcription factor p63 is a crucial regulator of epidermal development and stratification (Koster and Roop, 2007). Lechler and Fuchs (Lechler and Fuchs, 2005) showed that the deletion of p63 leads to an almost complete loss of asymmetrically dividing cells in the developing epidermis. In addition, p63 regulates cell cycle progression and proliferative potential (Truong et al., 2006; Koster and Roop, 2007; Beretta et al., 2005), which are both disturbed in keratinocytes of IGF-1R$^{epi-/-}$ and dko^{epi} epidermis (Fig. 8B and 9B&C). Thus regulation of p63 might provide a potential mechanism how IIS controls epidermal morphogenesis. To test whether loss of IIS has an effect on p63 activity we isolated primary keratinocytes from control and IGF-1R$^{epi-/-}$ newborn mice and performed p63 reporter assays. Transient transfection with a luciferase reporter that is repressed by p63 (pG13-Luc) (Hermeking et al., 1997; Yang et al., 1998) revealed a significant increase in reporter activity in IGF-1R deficient keratinocytes compared to control cells (Fig. 12A), suggesting a reversion of the repressive function of p63 upon loss of IGF-1R. In line with these data, transfection with a p63 transactivated reporter (BDS-2(3x)) (King et al., 2003) resulted in a decrease of reporter activity in IGF-1R$^{epi-/-}$ cells, again indicating a reduced p63 transcriptional activity (Fig. 12B). Furthermore, this inhibition could be reversed by expression of additional exogenous ΔNp63α. As both reporters consist mainly of p63 binding sites, this indicates a change of p63 activity on target gene regulation.

One possible explanation for these changes in p63 reporter activity in IGF-1R deficient keratinocytes would be a decrease in the expression of p63 itself. Thus, we asked whether p63 expression is altered upon loss of IIS. Realtime qPCR and Western blot analysis of primary keratinocytes revealed no change in total p63 RNA or protein expression in IGF-1R$^{epi-/-}$ cells (Fig. 12C&D). To assess whether the

altered p63 reporter assays also were reflected in functional p63 changes, we asked whether mRNA expression of endogenous p63 target genes was changed in IGF-1R$^{epi-/-}$ keratinocytes. Quantitative realtime PCR analysis on RNA isolated from primary keratinocytes revealed an increase in expression of e.g. *Sfn* (14-3-3σ), *Runx2* and *Tgfb1*, which are known p63 repressed targets in the absence of IGF-1R (Fig. 12C), whereas other known p63 targets, such as *Fgf2r* and *Cdkn1a*, were not altered. These data indicate loss of IIS induced alterations in p63 transcriptional activity on a subset of endogenous target genes, which is independent of changes in p63 expression in primary keratinocytes.

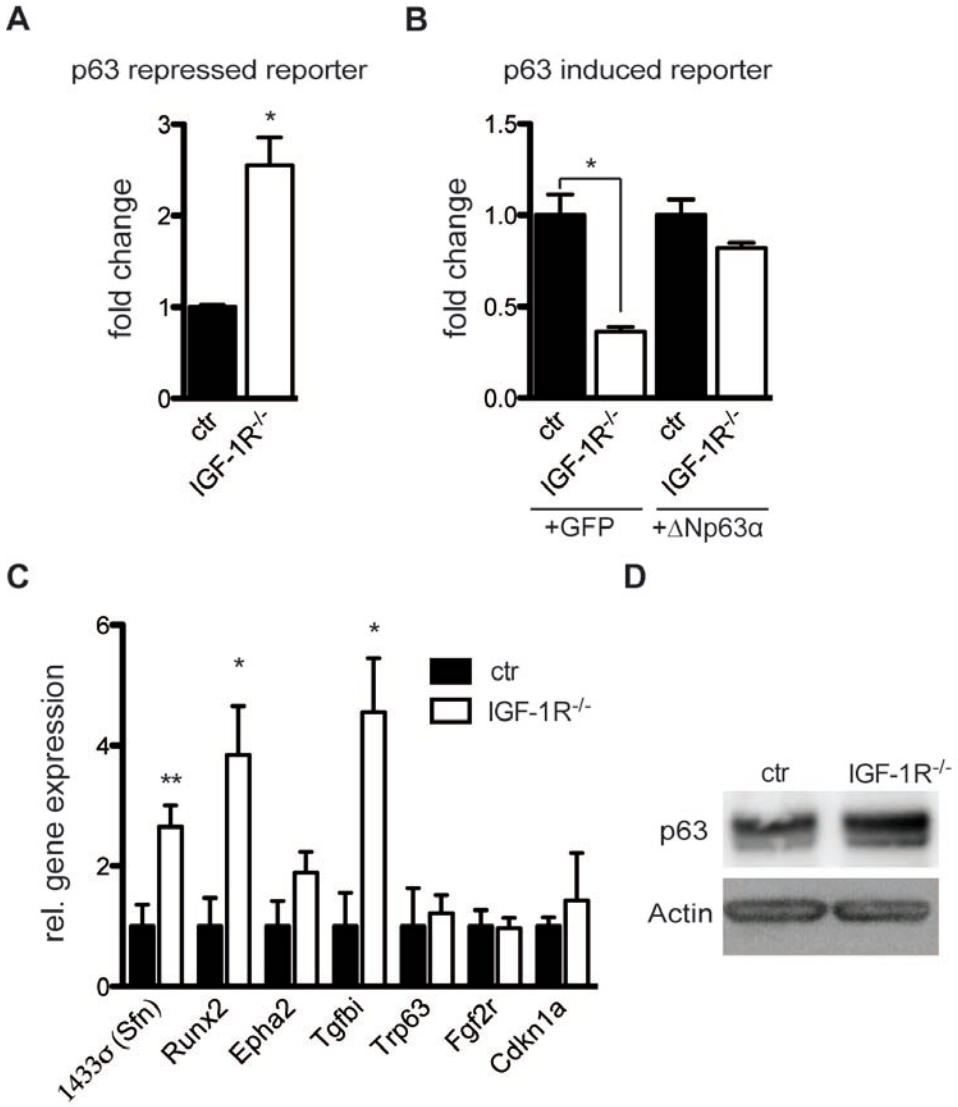


Figure 12: IGF-1 signaling negatively regulates p63 transcriptional activity in keratinocytes. (A) Luciferase reporter assay using the p63-repressed pG_{13} reporter in IGF-$R^{-/-}$ keratinocytes compared to control keratinocytes. Control keratinocytes were set to 1 and displayed is the mean of n=4/genotype independent experiments ± SEM. Statistical significance was tested by student's T-test and indicated as * p< 0.05. (B) Luciferase reporter assay using the p63-transactivated BDS-2(3x) luciferase gene reporter in IGF-$1R^{-/-}$ keratinocytes. The reporter was co-transfected with GFP or ΔNp63α to test for rescue capabilities of p63 on reporter activity. Control activity was set to 1 and displayed is the mean of n=4/genotype independent experiments ± SEM) Statistical significance was tested by student's T-test and indicated as * p< 0.05. (C) Quantitative realtime PCR showing relative gene expression of several putative p63 targets in control and IGF-$1R^{-/-}$ primary cultured keratinocytes. Control cells were set to 1 and displayed is the mean of n=3 independently isolated cell lines ± SEM. Statistical significance was tested by student's T-test and indicated as * p< 0.05 and **p<0.01. (D) Western blot analysis of p63 expression in primary control and IGF-$1R^{-/-}$ keratinocytes.

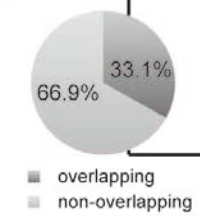
2.4. IIS and p63 share an overlapping gene expression set

To test whether these alterations in p63 transcriptional activity have functional consequences for p63 target gene expression *in vivo*, total gene expression analysis on RNA isolated from newborn epidermis of control and dko^{epi} mice was performed. The obtained expression data set was compared with published expression data sets of either mouse keratinocytes in which p63 was depleted by siRNA interference (Della Gatta et al., 2008) or E18.5 $p63^{-/-}$ whole skin data (Koster et al., 2006). Calculation of the hypergeometric distribution revealed a statistically highly significant overlap of the dko^{epi} gene expression set with both p63 regulated data sets (Fig. 13A). This percentage was in a similar range as the overlap of the p63 gene expression sets (Fig. 13B), thus suggesting that p63 and IIS regulate partially the same genes. More importantly, gene ontology terms related to epidermal development, such as regulation of cell proliferation and ectodermal development, were only significantly enriched in the overlapping gene sets of dko^{epi} with either the p63 KD keratinocytes

or the p63$^{-/-}$ E18.5 skin but not in the non-overlapping sets. These data indicate that p63 and IIS regulate a subset of similar target genes.

A

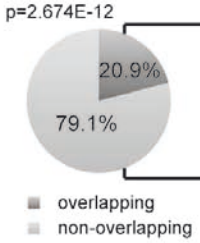
dko^{epi} newborn epidermis vs. p63 kd keratinocytes



GO Term	PValue	Fold Enrichment
regulation of cell proliferation	6,09E-06	2,385336638
enzyme linked receptor protein signaling	1,20E-05	3,041676842
positive regulation of developmental growth	1,35E-05	17,42051282
epidermis development	1,16E-04	3,925422222
ectoderm development	2,10E-04	3,6893066

GO Term	PValue	Fold Enrichment
fatty acid metabolic process	1,86E-06	2,902316309
regulation of transcription	3,67E-06	1,403252004
transcription	4,59E-06	1,462198652
regulation of transcription, DNA-dependent	6,35E-06	1,512095424
regulation of RNA metabolic process	7,67E-06	1,502015151

dko^{epi} newborn epidermis vs. p63$^{-/-}$ E18.5 skin

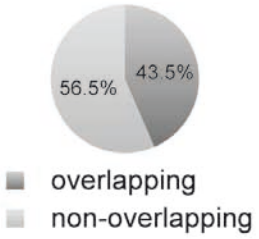


GO Term	PValue	Fold Enrichment
epidermis development	3,34E-09	7,464652361
ectoderm development	7,92E-09	7,015650715
epithelial cell differentiation	2,25E-08	7,11190202
epithelium development	9,36E-07	4,088687582
regulation of cell proliferation	1,70E-06	2,92671953

GO Term	PValue	Fold Enrichment
fatty acid metabolic process	1,37E-07	3,434782609
transcription	1,36E-06	1,550095503
regulation of transcription, DNA-dependent	2,00E-06	1,6094513
leukocyte activation	3,69E-06	2,885844749
regulation of transcription	3,85E-06	1,451694242

B

p63$^{-/-}$ E18.5 skin vs. p63 kd keratinocytes



Common GO terms in arrays of p63-/- E18.5 skin and p63 kd keratinocytes

GO Term	PValue	Fold Enrichment
epidermis development	3,23E-12	3,076759789
ectoderm development	4,81E-12	2,963378356
response to organic substance	1,21E-10	1,848474039
response to endogenous stimulus	9,47E-10	2,125876414
regulation of cell proliferation	2,84E-09	1,738414892

Unique GO terms in p63-/- E18.5 skin array

GO Term	PValue	Fold Enrichment
chemical homeostasis	4,15E-14	2,060255643
response to wounding	4,85E-13	1,990284697
cellular chemical homeostasis	3,21E-11	2,094117647
homeostatic process	6,52E-11	1,7249413
muscle organ development	9,56E-11	2,499646657

Unique GO terms in p63 kd keratinocytes array

GO Term	PValue	Fold Enrichment
regulation of programmed cell death	1,24E-11	1,512121392
regulation of apoptosis	1,37E-11	1,51365262
regulation of cell death	1,80E-11	1,506555301
protein localization	7,50E-10	1,44139005
establishment of protein localization	2,93E-08	1,427116006

Figure 13: Overlap in global gene expression data of dko^{epi} epidermis and p63 deficient keratinocytes/skin. (A) Comparison of gene expression microarray analysis of newborn dko^{epi} epidermis with arrays of p63 knockdown keratinocytes (upper panel) (Della Gatta et al., 2008) or p63$^{-/-}$ E18.5 skin (Koster et al., 2006) show overlap in gene sets (Pie charts). The significance of the array overlap was calculated using hypergeometric distribution algorithm (p-values). Analysis of gene ontology (GO) terms for overlapping genes and non-overlapping GO terms is displayed in the table right to the pie chart. (B) Pie charts showing the percentage of overlapping and non-overlapping genes of the p63$^{-/-}$ E18.5 skin gene set (Koster et al., 2006) with the p63 kd keratinocytes gene expression set (Della Gatta et al., 2008). Tables show GO terms for the overlapping genes and the unique genes for either of the p63 gene sets.

2.5. Epidermal IIS regulates p63 target gene expression

The microarray comparison indicated that IIS and p63 regulate the same transcription targets. To confirm this we tested specific targets identified in the dko^{epi} newborn epidermis array by realtime qPCR and found an increased expression of several known p63 repressed target genes, such as *Sfn* (14-3-3σ), whereas the expression of positively regulated targets, such as *Keratin 15*, were decreased (Fig. 14A). In line with the *in vitro* data , the expression of other known p63 targets, such as *Fgf2r*, was not affected by the loss of IR/IGF-1R (Fig. 14A). Interestingly, the global gene expression analysis revealed increased expression of genes that are a part of the epidermal differentiation complex (EDC), which is known to be indirectly regulated by p63 (Fessing et al., 2011). Realtime PCR analysis confirmed the strong upregulation of several of these EDC members, e.g. the late cornified envelope (*Lce*) protein families and the small proline rich (*Sprr*) (Fig. 14C).

As the phenotype induced by loss of epidermal IIS signaling becomes first obvious at E16.5, we also tested whether the expression of different p63 target genes was changed in the epidermis at this developmental stage. Realtime PCR analysis showed that several targets that are repressed by p63 activity, such as *Sfn* (14-3-3σ)

and *Runx2* were upregulated, whereas other targets, such as *Keratin 15* or *Tgfbi*, were downregulated in the epidermis of IGF-1R$^{epi-/-}$ mice (Fig. 14B). As expected, RNA expression of p63 at E16.5 was not reduced and even slightly increased in IGF-1R$^{epi-/-}$ epidermis (Fig. 14B), similar to the changed RNA and protein expression observed *in vitro* (Fig. 12C&D). Furthermore, immunofluorescence (IF) analysis of skin sections revealed that the localization and staining intensity of p63 was unaltered in the epidermis of E16.5 IGF-1R$^{epi-/-}$ and dko^{epi} mice (Fig. 14D).

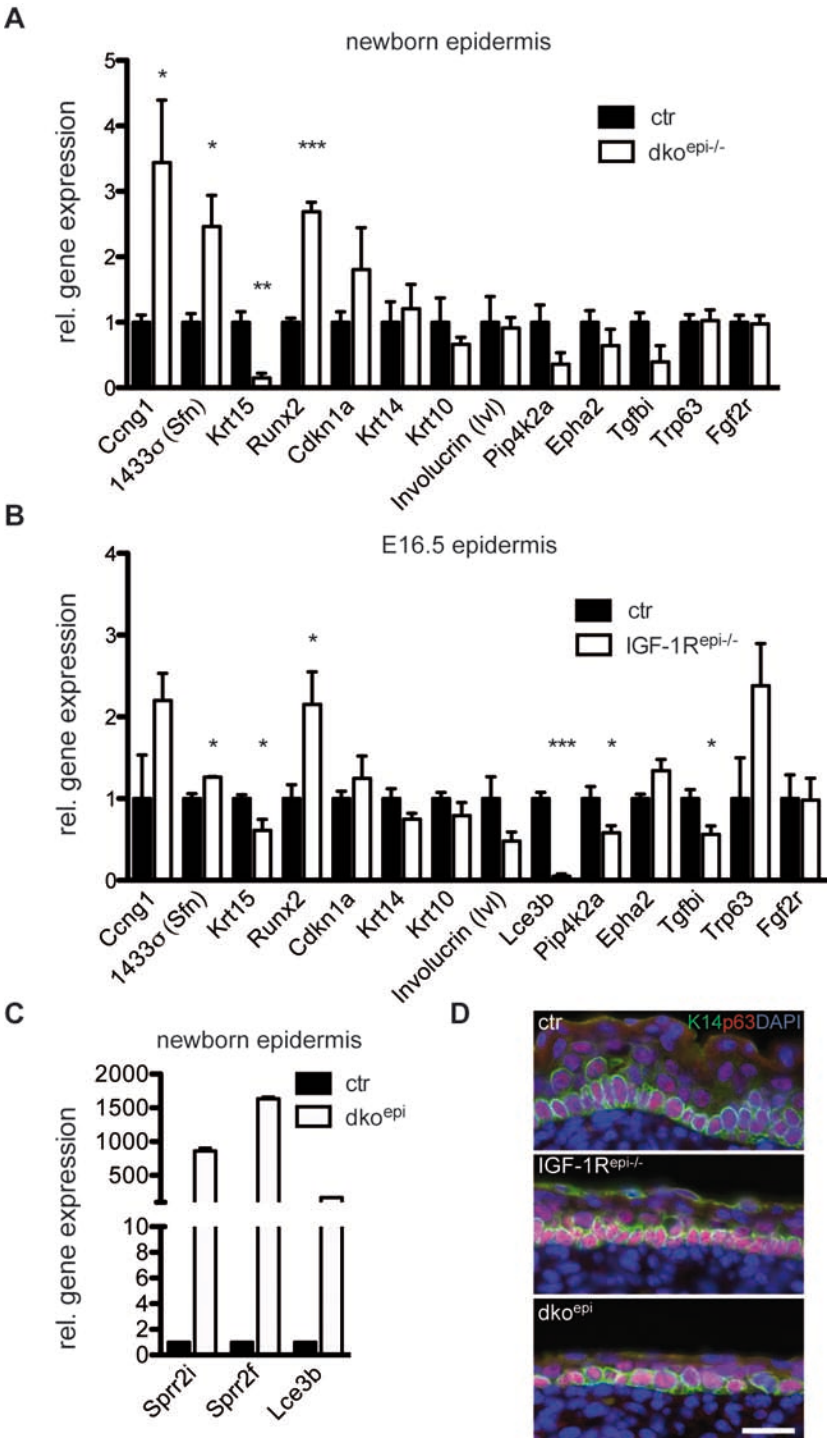


Figure 14: Insulin/IGF-1 signaling regulate the expression of p63 target genes. (A) Quantitative realtime PCR analysis analyzing relative gene expression of p63 regulated genes in (ctr) and dko^{epi} newborn epidermis. Displayed is the mean of n=3 newborn epidermis/genotype ± SEM. Control was set to 1. (B) Quantitative realtime qPCR analysis to determine relative gene expression of indicated p63 regulated genes in control and IGF-1R$^{epi-/-}$ E16.5 epidermis. Displayed is the mean of n=3 embryos/genotype ± SEM. (C) Quantitative realtime PCR analysis showing relative gene expression of selected EDC genes in control (ctr) and dko^{epi} newborn epidermis. Displayed is the mean of n=3

newborn epidermis/genotype ± SEM. Control was set to 1. (D) Immunofluorescence analysis of p63 (red) and keratin 14 (green) in the epidermis of E16.5 embryos showing p63 localization in control (ctr), IGF-1R$^{epi-/-}$ and dko^{epi} mice.

2.6. Binding of p63 to target promoters is not altered

The altered expression of a subset of p63 targets genes observed in vitro and in the developing epidermis *in vivo* may be explained by a reduced binding of p63 to p63 consensus elements in the promoters/enhancers of these genes. To test this we identified p63 consensus binding sequences in promoters and enhancers of a subset of these targets. The conserved p63-binding element for *Fgf2r* was already described (Ferone et al., 2011) and as its expression is not changed upon IIS alteration served as control. For mouse *Sfn* (14-3-3σ), *Cdkn1a* and *Runx2* we identified new p63 consensus sites in the promoter regions by comparing their promoter region to the published human p63 binding sites identified in these genes using chromatin-immunoprecipitations (ChIP) followed by next generation sequencing (ChIP-seq) data in human keratinocytes (Kouwenhoven et al., 2010). Using ChIP assays followed by quantitative PCR analysis (ChIP-qPCR), we next asked if p63 binding to these sites was changed after loss of IGF-1R. Surprisingly, similar amounts of p63 protein were bound to p63 consensus sites in the promoters of *Runx2*, *Sfn* (14-3-3s), *Cdkn1a* and, as a negative control, *Fgfr2* in IGF-1R$^{-/-}$ and control primary keratinocytes (Fig. 15). Thus, p63 binding to the p63 consensus binding sites was not affected by the loss of IGF-1R in keratinocytes despite the change in expression of these targets. Together, these data suggest that IIS regulates p63 transcriptional activity independent of alterations in p63 expression and promoter binding.

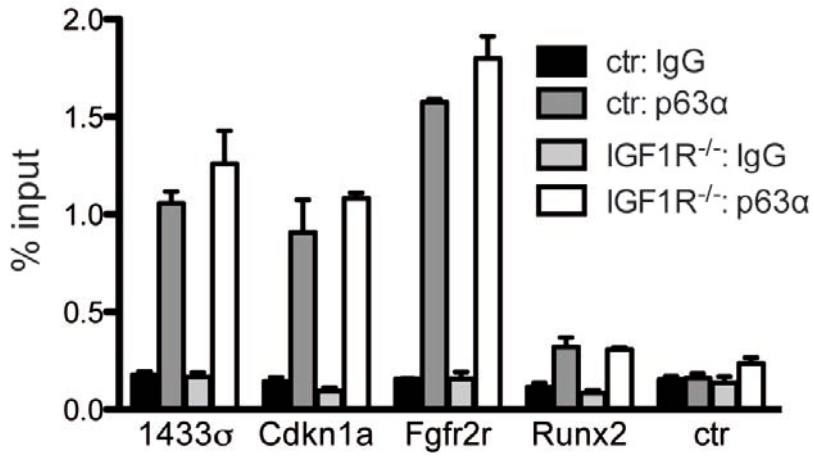


Figure 15: No change in binding of p63 to consensus sites in promoters/enhancers of regulated genes. Chromatin immunoprecipitation–qPCR (ChIP-qPCR) analysis in primary keratinocytes showing binding of p63 to p63 binding regions in different target promoters of control (ctr) and IGF-1R$^{epi-/-}$ keratinocytes. Shown is one example that represents the mean of three technical qPCR replicates ± SD of three independent experiments.

2.7. IIS regulated FoxO transcription factors are expressed during epidermal development and in keratinocytes *in vitro*

FoxO transcription factors are key downstream targets through which IIS exerts its transcriptional regulation and thereby its biological effects (Calnan and Brunet, 2008; Eijkelenboom and Burgering, 2013). Interestingly, FoxOs were shown to regulate transcriptional activity of other transcription factors, which did not seem to require FoxO binding to its consensus site in promoters (Nemoto et al., 2004; Jensen et al., 2011). Therefore, FoxOs may serve as potential candidates to mediate IIS control of p63 target genes and p63 activity in keratinocytes.

Very little is known on the expression of FoxO isoforms in the developing epidermis. We therefore first investigated the expression of FoxO mRNA in the developing epidermis and in keratinocytes. Realtime qPCR analysis indicated that three members of the FoxO family, namely FoxO1, FoxO3 and FoxO4, were expressed in E16.5 epidermis (Fig. 16A) and in primary keratinocytes (Fig. 16B). We additionally

detected FoxO6 mRNA in E16.5 epidermis, but as IIS does not regulate nuclear shuttling of FoxO6 (Jacobs et al., 2003), we did not further analyze this member. Western blot analysis for FoxO1 and FoxO3 also showed protein expression at E16.5 and in newborn epidermis as well as in primary keratinocytes (Fig. 16C). For FoxO4 a specific band could be detected in primary keratinocytes but not in E16.5 epidermis despite RNA expression at this developmental stage (Fig. 16A).

Thus at least two of the four FoxO transcription factors, FoxO1 and FoxO3, are expressed on the RNA and the protein level in the developing epidermis at E16.5, whereas primary keratinocytes express FoxO1, -3 and -4.

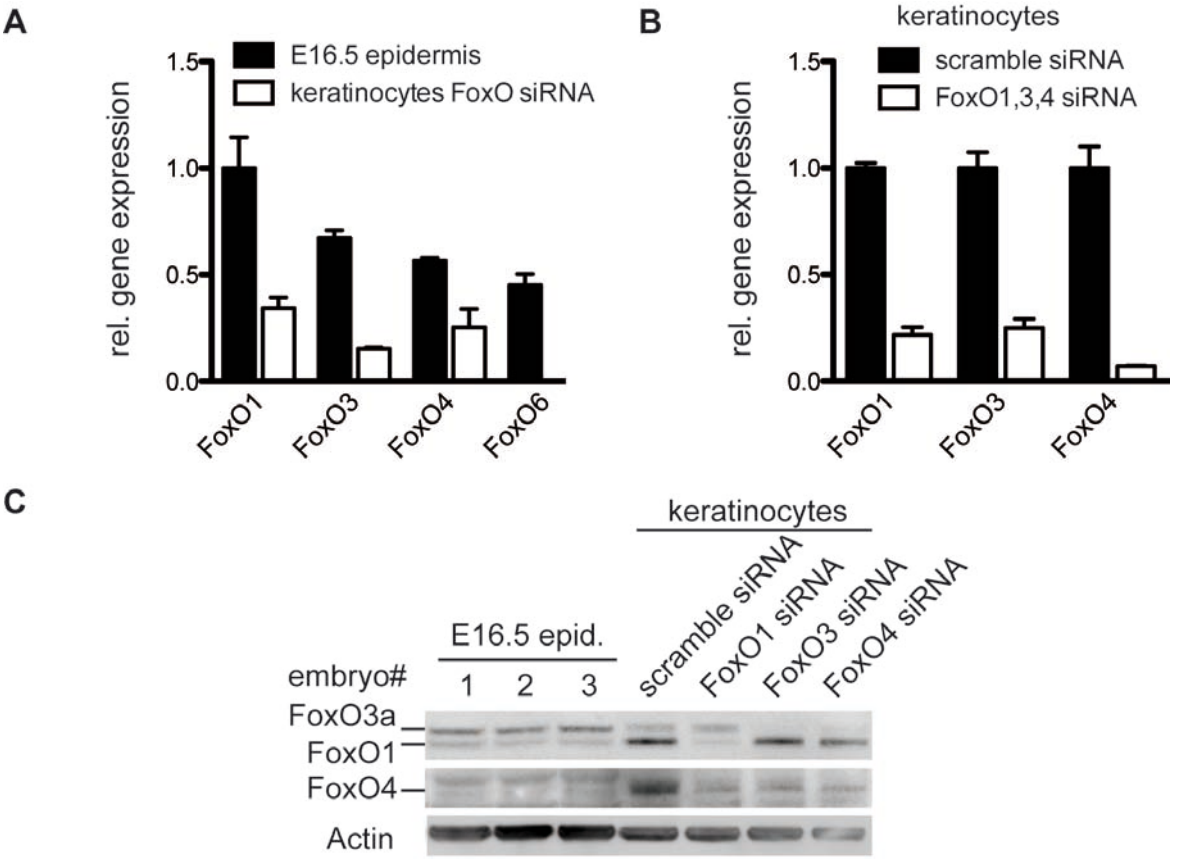


Figure 16: FoxO transcription factors are expressed in E16.5 epidermis and primary keratinocytes. (A) Quantitative realtime PCR analysis of the mammalian FoxO family members in RNA isolated from E16.5 epidermis and in RNA isolated from keratinocytes that were transfected with a combination of siRNAs for FoxO1/3/4 served as negative control. Expression of FoxO1 was set to 1.

Displayed is the mean of n=3 embryos/knockdown cell lines ± SEM. (B) Quantitative realtime PCR analysis of FoxOs in primary murine keratinocytes transfected with siRNAs targeting FoxO1/3/4 or scrambled siRNA as control. Scrambled siRNA cell lines were set to 1. Displayed is the mean of n=3 cell lines ± SEM. (C) Western blot analysis of FoxO family member expression in E16.5 epidermis and primary keratinocytes. Specificity of FoxO1 and FoxO3 antibody was tested by siRNA knockdown.

2.8. IIS regulates nuclear translocation of FoxOs in keratinocytes

IIS activates the Akt kinase, which phosphorylates FoxO proteins at 3 conserved sites resulting in nuclear export of FoxOs (Calnan and Brunet, 2008; Eijkelenboom and Burgering, 2013). We therefore asked whether more FoxO remains in the nucleus and is transcriptional active upon loss of IIS. First, we transfected primary keratinocytes with the FoxO binding site only reporter 6xDBE and observed a two-fold increase in luciferase activity in IGF-R deficient cells compared to control cells (Fig. 17A). Nuclear fractionation experiments of control and IGF-1R$^{-/-}$ keratinocytes revealed an increase in levels of nuclear FoxO1 in cells that lack the IGF-1R, although no change in total FoxO1 or FoxO3 protein expression were observed in these cells (Fig. 17B). Importantly, using western blot analysis, we also detected less phospho-FoxO1 in E16.5 IGF-1R$^{epi-/-}$ compared to control epidermis, indicating a loss of IIS-stimulated Akt phosphorylation of FoxO and more nuclear FoxO localization (Fig. 17C). Quantification of this phosphorylation western blot revealed a three-fold decrease of phospho-FoxO1 in IGF-1R$^{epi-/-}$ epidermis (Fig. 17D). RNA expression of FoxO1 and FoxO3 was also not changed (Fig. 17E), in agreement with the unaltered protein level. In conclusion, loss of IIS signaling promotes nuclear FoxO activity.

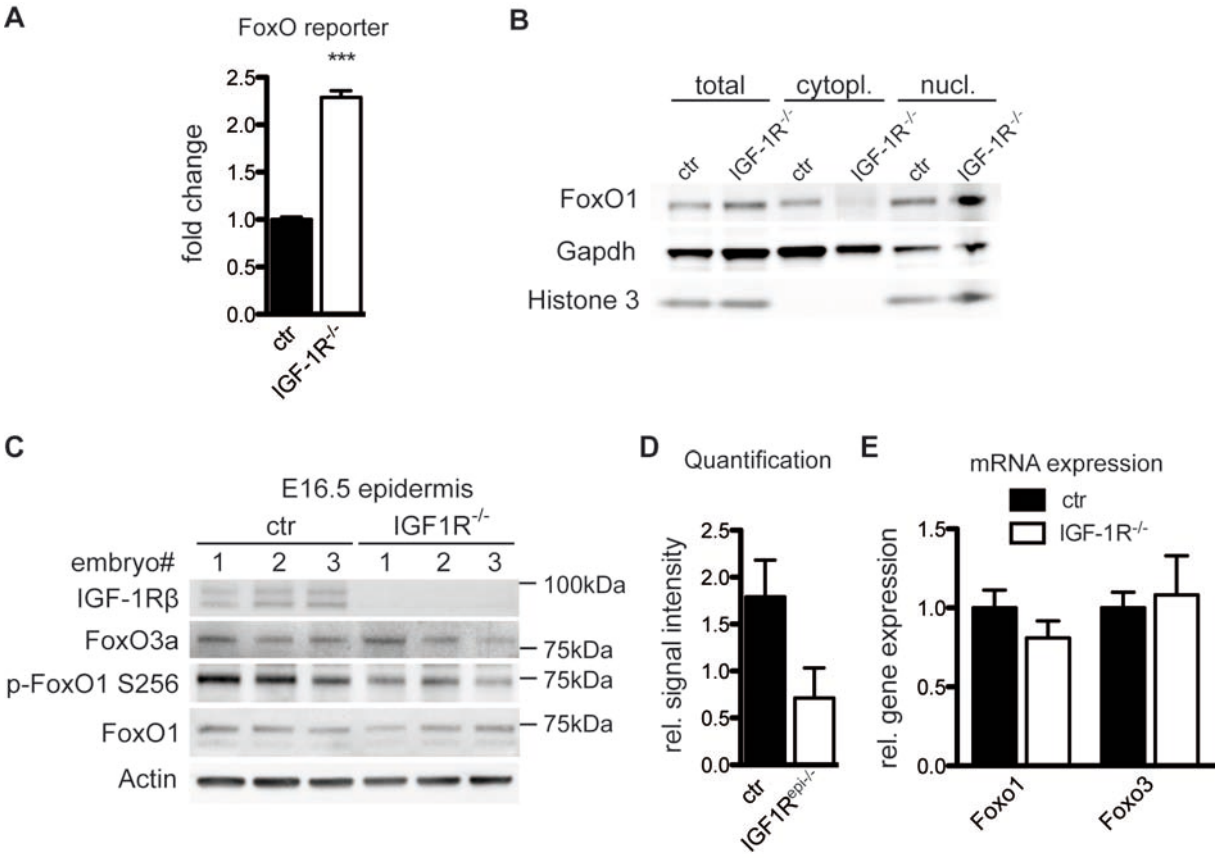


Figure 17: Epidermal FoxO transcription factors are negatively regulated by IIS. (A) Luciferase reporter analysis of control (ctr) and IGF-1R$^{-/-}$ keratinocytes using the FoxO reporter 6xDBE. Control was set to 1. Shown is the mean of n=4 independent experiments ± SEM. Statistical significance was tested by student's T-test. (B) Western blot analysis of cell fractionations from control and IGF-1R$^{-/-}$ keratinocytes tested for FoxO1 expression/localization in cytoplasmic or nuclear fractions. Gapdh served as cytoplasmic and histone 3 as nuclear control. (C) Western blot analysis showing the expression of FoxO1 and FoxO3 in E16.5 epidermis of control (ctr) and IGF-1R$^{epi-/-}$ mice. Phosphorylation at Serin 256 determines the cytoplasmic localization of FoxO1. (D) Quantification of phospho-FoxO1 intensities in (C) normalized to total FoxO1 with control set as 1. Displayed is the mean of n=3 E16.5 epidermis/genotype ± SD. (C) Quantitative realtime PCR analysis of E16.5 epidermis for FoxO1 or FoxO3 expression in control (ctr) and IGF-1R$^{epi-/-}$ mice. Control was set as 1 and displayed is the mean of n=3 embryos/genotype ± SEM.

2.9. Nuclear FoxO1-ADA impairs p63 activity

We next asked whether FoxO is able to regulate p63 activity. To uncouple FoxO regulation from IIS/Akt signaling the three Akt-phosphorylation sites were mutated resulting in a constitutive nuclear FoxO (FoxO1-ADA), which thus mimics loss of IIS. First, we asked whether this FoxO mutant affected p63 luciferase reporter activity. We transiently transfected primary keratinocytes with the p63-transactivated reporter (BDS-2.3x) together with either FoxO1-ADA or FoxO1-WT cDNA (wild-type FoxO1) as a control, as this is excluded from the nucleus due to Insulin/IGF-1 in the serum. Co-transfection of the reporter with FoxO1-ADA resulted in a decrease in reporter activity compared to co-transfection with FoxO1-WT (Fig. 18A). In addition, upon transfection of keratinocytes with the p63-repressed reporter (pG13) resulted in an increase in reporter activity when co-transfected with FoxO1-ADA compared to FoxO1-WT (Fig. 18B). These data suggest that nuclear FoxO counteracts p63 activity in primary keratinocytes similar to the loss of IIS.

To examine whether FoxO-mediated regulation of p63 reporters required p63 expression, we used transfected chinese hamster ovarian (CHO) cells as these cells do not express endogenous p63 in combination with the p63-transactivated reporter Whereas FoxO1-ADA transfection alone did not induce p63 reporter activity above GFP transfected background activity, ΔNp63α transfection strongly increased reporter activity. This increase was reversed upon co-transfection of FoxO1-ADA and ΔNp63α (Fig. 18C). Together, these data indicate that FoxO cannot directly bind to p63 DNA consensus sites to induce reporter activity but requires the presence of p63.

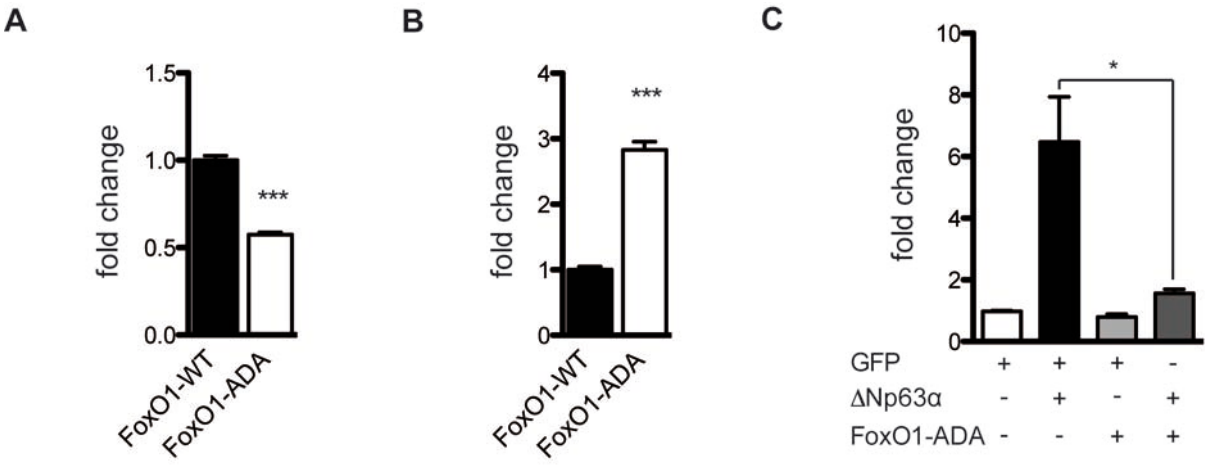


Figure 18: Nuclear FoxO counteracts p63 activity. (A) Transient transfection luciferase reporter analysis of p63-induced BDS-2(3x) reporter activity upon co-transfection with wild type FoxO1 (FoxO1-WT) or FoxO1-ADA in primary keratinocytes. Displayed is the mean of n=4 independent experiments ± SEM and statistical significance was tested by student's T-test and indicated as ***p<0.001. (B) Luciferase reporter analysis of p63-repressed reporter pG13 activity in primary keratinocytes, which were co-transfected with wild-type FoxO1 (FoxO1-WT) or FoxO1-ADA. Displayed is the mean of n=4 independent experiments ± SEM and statistical significance was tested by student's T-test and indicated as ***p<0.001. (C) Transient luciferase reporter analysis of the p63-activated (BDS-2(3x)) reporter in CHO cells, which were co-transfection with GFP/FoxO1-ADA, GFP/ΔNp63α or FoxO1-ADA/ΔNp63α. Shown is the mean of n=4 independent experiments ± SEM. Statistical significance was tested by student's T-test and indicated as * p< 0.05.

2.10. FoxO regulation of p63 activity is independent of FoxO-DNA interaction

Our observations suggested that the regulation of p63 activity was independent of FoxO's ability to bind DNA. To directly test this we introduced a point mutation in the DNA-binding site of FoxO1-ADA resulting in a constitutive nuclear FoxO1 that cannot bind DNA (FoxO1-ADA-ΔDBD). FoxO-reporter assays showed that this mutant was unable to activate luciferase activity whereas FoxO-ADA strongly activated the reporter in comparison to FoxO1-WT (Fig. 19A). We then tested whether the FoxO1-ADA-ΔDBD mutant was able to regulate the p63-transactivated reporter (BDS-2(3x)) in primary keratinocytes. Similar to FoxO1-ADA, co-transfection of FoxO1-ADA-

ΔDBD reduced p63-transactivated reporter activity in keratinocytes, although the effect was not as strong as for FoxO1-ADA (Fig. 19B). In addition, in CHO cells transfection of FoxO1-ADA-ΔDBD with p63 reduced the p63 reporter activity to a similar extent as FoxO1-ADA (Fig. 19C). Again, this regulation required the presence of p63, as FoxO1-ADA-ΔDBD expression alone had no effect on reporter activity. These data suggest that FoxO modulates p63 activity independent of its ability to bind DNA.

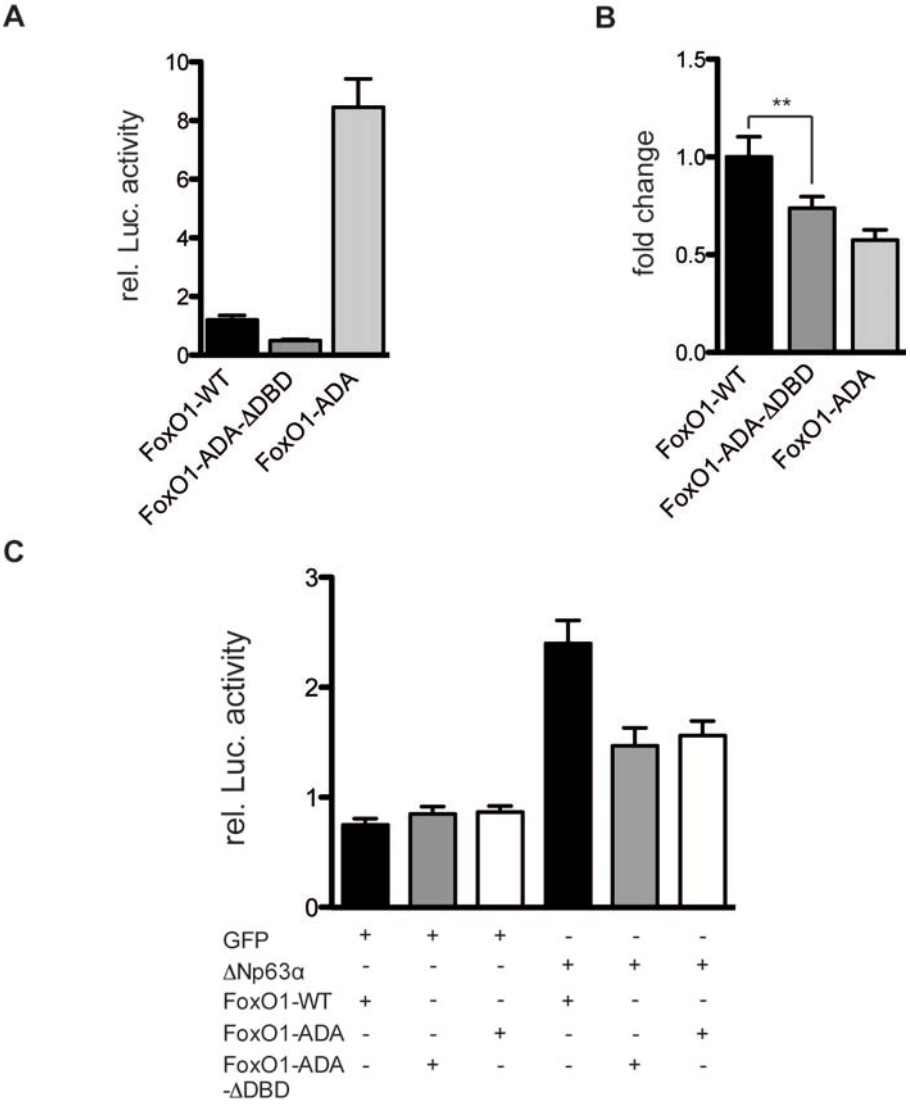


Figure 19: Nuclear FoxO negatively regulate p63 transcriptional activity independent of DNA binding. (A) FoxO-luciferase reporter (6xDBE) transfection experiments in primary keratinocytes

transfected with wild-type FoxO1 (FoxO1-WT), constitutive nuclear FoxO1-ADA and the nuclear mutant FoxO1-ADA-ΔDBD. Displayed is one example of n=2 experiments ± SD of 3 technical replicates. (B) Transient Luciferase reporter assays using the p63-activated reporter BDS-2(3x) in combination with FoxO1-WT, FoxO1-ADA or the DNA-binding deficient mutant FoxO1-ADA-ΔDBD in primary keratinocytes. Displayed is the mean of n=4 independent experiments ± SEM. Statistical significance was tested by student's T-test and indicated as **p<0.01. (C) Luciferase reporter analysis in CHO cells, which were co-transfected with the p63-activated reporter BDS-2(3x) and p63/FoxO1 expression plasmids in the following combinations: GFP/FoxO1-WT, GFP/FoxO1-ADA, GFP/FoxO1-ADA-ΔDBD, ΔNp63α/FoxO1-, ΔNp63α/FoxO1-ADA and ΔNp63α/FoxO1-ADA-ΔDBD. Shown is the mean of n=3 technical replicates ± SD as an example of n=3 independent experiments.

2.11. FoxO interacts with p63

The observation that FoxO-mediated suppression of p63 reporter activity requires ΔNp63α expression and is independent of FoxO-DNA binding suggested a direct interaction of FoxO with p63. We therefore performed co-immunoprecipitation (Co-IP) experiments using CHO cells and transiently transfected them with either GFP in combination with FoxO1-ADA or Flag-ΔNp63α, or with FoxO1-ADA together with Flag-ΔNp63α experiments. Using an antibody against the Flag-tag FoxO1 was co-precipitated with Flag-ΔNp63α whereas no FoxO1 was precipitated by control IgG (Fig. 20A). Vice versa, only antibodies to FoxO1-ADA but not control IgG precipitated ΔNp63α (Fig. 20B), thus providing evidence that overexpressed FoxO1-ADA and ΔNp63α can interact in a heterologous cell system. To examine if endogenous p63 and FoxOs could interact and whether loss of IGF-1R would increase this interaction as more FoxO is present in the nucleus (see Fig. 17A&B), we performed Co-IPs in keratinocytes after formaldehyde fixation followed by western blot analysis (ChIP-western blot). More endogenous FoxO1 was precipitated in IGF-1R$^{-/-}$ keratinocytes compared to control using antibodies against endogenous p63α (Fig. 20C), thus

confirming the CHO data. Finally, to address whether FoxO and p63 also interact *in vivo* in the epidermis, we sued epidermal splits of newborn IGF-1R$^{epi-/-}$ mice, which additionally expressed GFP-tagged FoxO1 (FoxO1-GFP) in the epidermis. Using GFP antibodies more p63 was co-precipitated with FoxO-GFP in IGF-1R$^{epi-/-}$ epidermis compared to control epidermis (K14-Cre, IGF-1R$^{fl/+}$, FoxO1-GFP) (Fig. 20D). Taken together, these results show that FoxO interacts with p63 both in vivo and in vitro and that loss of IIS leads to an increase in this interaction.

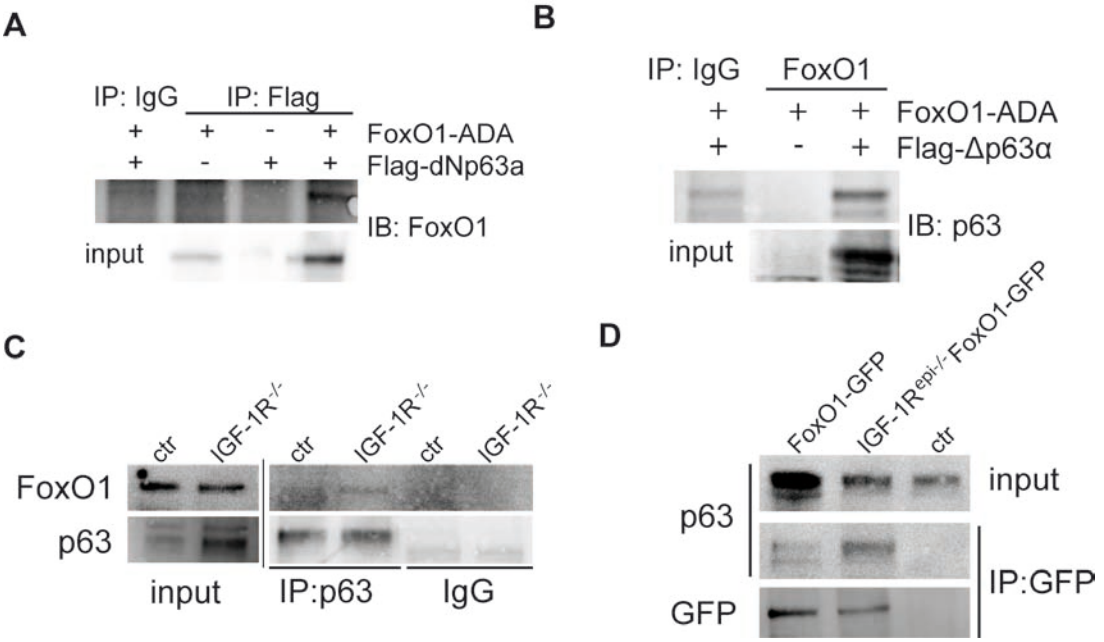


Figure 20: FoxO1 interacts with p63 in heterologous overexpression experiments, in keratinocytes and *in vivo*. (A and B) Co-immunoprecipitation (Co-IP) analysis in CHO cells transiently transfected with FoxO1-ADA and Flag-ΔNp63α tested for (A) immunoprecipitation (IP) of FoxO1-ADA by ΔNp63α using Flag antibodies and vice versa (B) FoxO1 antibodies precipitate FoxO1-ADA and p63. Shown is one representative experiment of three independent ones. (C) Co-IP analysis of endogenous FoxO1, which was immune-precipitated with p63 antibodies from primary IGF-1R$^{-/-}$ but control keratinocytes. (D) Co-IP experiment in newborn epidermis of control and IGF-1R$^{epi-/-}$ mice, which express epidermal FoxO1-GFP. GFP antibodies were used for IP and detected was p63 by western blot.

2.12. IIS signaling regulates recruitment of FoxO to p63-DNA consensus binding sites

The data thus far would predict that loss of IIS promotes association of FoxOs with p63 at p63 binding sites in endogenous promoters of regulated genes. To test whether more FoxO is associated with these p63 consensus sites, we performed ChIP-qPCR experiments using FoxO1 antibodies. In line with our prediction, we observed that upon loss of IGF-1R FoxO1 was enriched 2- to 3-fold on p63 consensus sequences in the promoters of altered genes, such as *Sfn* (14-3-3σ) and *Runx2* (Fig. 21). As a negative control we used the p63-binding site in the promoter of *Fgf2r*, as expression of this gene was not altered upon loss of IIS. As expected no increase was observed in FoxO1 binding to this promoter (Fig. 21). Together with the Co-IP data these results indicate that FoxOs interact with p63 on endogenous p63 binding sites to regulate the expression of these targets.

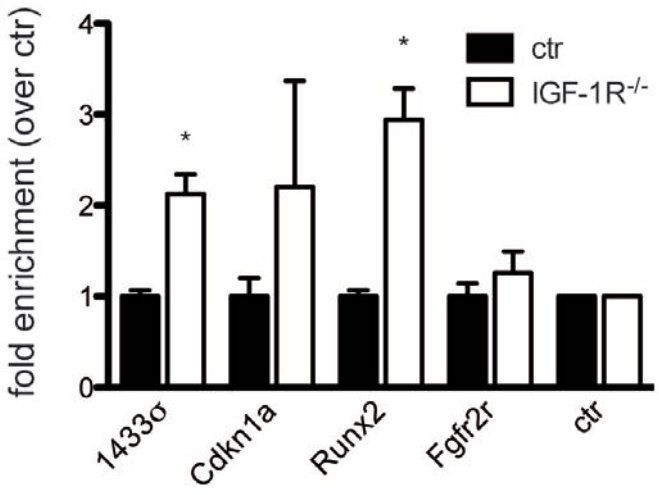


Figure 21: Epidermal FoxOs binds p63 at consensus site. Chromatin immunoprecipitation (ChIP)-qPCR analysis in primary mouse control and IGF-1R$^{-/-}$ keratinocytes using FoxO1 antibodies for precipitation. Precipitates were analyzed with primers for p63 binding sites in promoter regions of known p63 target genes. Results were normalized to a negative binding region and are shown as enrichment over control keratinocyte IP, which was set to 1. Shown is the mean of n=3 independent

experiments ± SD (n=2 for Fgf2r). Statistical significance was tested by student's T-test and indicated as * p< 0.05.

2.13. FoxO binding to p63 is important for target gene regulation

The next question that was addressed was whether the increased binding of FoxO to the p63 consensus promoters of p63 targets is indeed responsible for their altered expression upon loss of IIS. We therefore performed siRNA-mediated knockdown of FoxO-1,-2 and -4 in IGF-1R$^{-/-}$ keratinocytes and asked whether downregulation of FoxOs would reverse the increased expression of these genes using realtime qPCR. FoxO siRNA-mediated knockdown but not scrambled siRNA downregulated expression of p63 target genes which showed increased expression upon loss of IIS (see Fig. 14), such as *Sfn* (14-3-3σ) and *Runx2* (Fig. 22A). No significant difference was observed upon FoxO knockdown in *Fgf2r* expression, in agreement with the fact that loss of IIS signaling does not alter expression of this gene. Most importantly, FoxO siRNAs did no affect mRNA expression of these targets in control cell (Fig. 22B). These data indicate that the increased expression of these targets upon loss of IGF-1R is a direct consequence of increased nuclear FoxO activity.

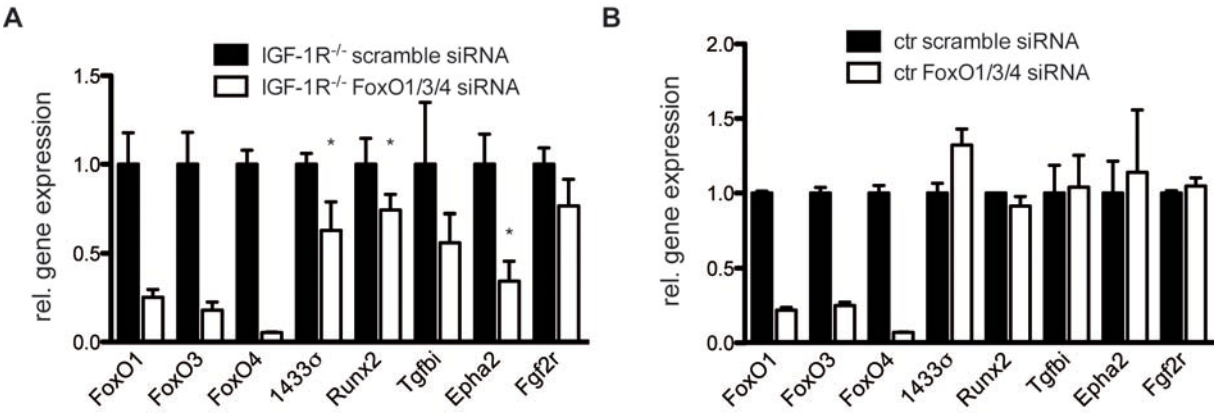


Figure 22: Epidermal FoxOs are important regulators of p63 transcriptional activity in keratinocytes. (A and B) Realtime PCR analysis of primary mouse (B) control and (A) IGF-1R$^{-/-}$ keratinocytes transiently transfected with either scrambled siRNA or combined knockdown of

FoxO1/3/4 using smart pool siRNAs to each of the FoxOs. Tested were p63 target genes, which were regulated in IGF-1R$^{epi-/-}$ E16.5 epidermis (see Fig. 14B). Shown are the mean of n=4 independent experiments ± SEM. Significance was tested using student's T-test and is indicated as *p<0.05.

2.14. Overexpression of FoxO1-DN does not rescue IGF-1R$^{epi-/-}$ phenotype

The data thus far indicate that in the epidermis FoxO regulates p63 activity downstream of IIS independent of FoxO's ability to bind DNA. If also true *in vivo*, then the prediction would be that epidermal expression of a dominant negative FoxO1 mutant that only consists of the DNA interaction domain (FoxO1-DN) would not interfere with stratification and this mutant would not be able to rescue the phenotypes caused by IGF-1R loss. We hypothesized that this mutant would occupy all accessible FoxO consensus sites and thereby inhibit the endogenous FoxO-DNA binding-dependent function in control and/or IIS deficient epidermis.

We first tested whether this mutant would alter p63 reporter activity in transiently transfected CHO cells. Surprisingly, FoxO1-DN also suppressed the p63-induced activity of the p63-transactivated reporter (Fig. 23A), suggesting that the DNA binding domain might be involved in the FoxO-p63 interaction.

To further assess the ability of FoxO1-DN to rescue the loss of IGF-1R, we crossed mice that carry a flox-stop-flox cassette followed by a FoxO1-DN-IRES-GFP cassette in the Rosa26 locus (Belgardt et al., 2008) with Keratin14-Cre mice (Hafner et al., 2004) and with the IGF-1R$^{fl/fl}$ mice. We isolated primary keratinocytes of these mice and tested the FoxO1-DN function by FoxO reporter assays. As expected the FoxO reporter activity was induced by the loss of IGF-1R and the expression of FoxO1-DN was able to rescue this effect (Fig. 23B). This shows that the FoxO DNA-binding mutant is indeed dominant negative towards DNA-binding dependent FoxO transcriptional activity.

More importantly, histological investigations of newborn skin sections revealed that the expression of FoxO1-DN did not rescue the IGF-1R$^{epi-/-}$ phenotype whereas FoxO1-DN expression alone had no effect on epidermal morphogenesis (Fig. 23C).

In summary, these data suggest that FoxO binding to FoxO consensus sites is not important for the developmental phenotype observed in IGF-1R$^{epi-/-}$ mice.

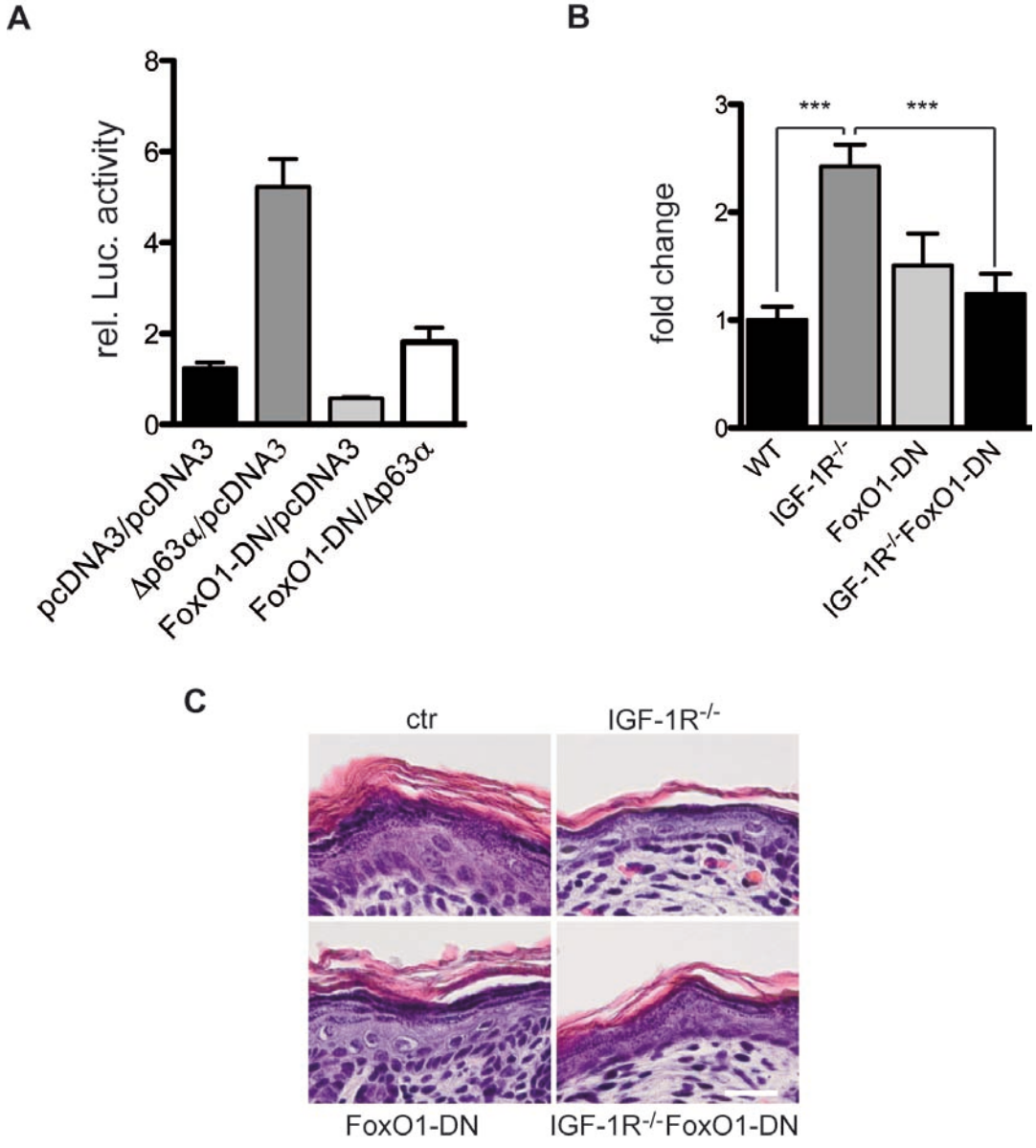


Figure 23: Epidermal expression of dominant negative FoxO1 does not rescue IGF-1R$^{epi-/-}$ phenotype. (A) Luciferase reporter analysis in CHO cells using the p63 induced BDS-2(3x) reporter, which was co-transfected with FoxO1-DN, ΔNp63α or pcDNA3 as transfection control. Shown is the mean of n=3 technical replicates ± SD as an example of n=2 independent experiments. (B) Transient

luciferase reporter analysis of FoxO reporter (6xDBE) activity in keratinocytes isolated from control, IGF-1R$^{epi-/-}$, FoxO1-DN and IGF-1R$^{epi-/-}$/FoxO1-DN newborn mice. Displayed is the mean of n=4 independent experiments ± SEM. Statistical significance was tested by student's T-test and indicated as ***p<0.001. (C) H&E staining of skin sections of control, IGF-1R$^{epi-/-}$, FoxO1-DN and IGF-1R$^{epi-/-}$/FoxO1-DN newborn mice. Scale bar is 25 μm.

2.15. Perinatal death of mice expressing epidermal FoxO1-ADA

As nuclear FoxO1-ADA is able to bind p63 and thereby inhibit the function p63, we hypothesized that the epidermal phenotype of mice overexpressing epidermal FoxO1-ADA would resemble the phenotype of p63$^{-/-}$ mice. To test this we crossed K14-Cre mice (Hafner et al., 2004) with mice carrying the flox-stop-flox cassette followed by the FoxO1-ADA-IRES-GFP in the Rosa26 locus (Stohr et al., 2011). Epidermal FoxO1-ADA expression resulted in a fragile, translucent skin and perinatal death (Fig. 24A) caused by massive dehydration (not shown). Histochemical analysis of these newborn mice revealed a striking hypoplastic epidermis with only one or two layers of keratinocytes (Fig. 24B). Immunohistochemical analysis of different embryonic stages using a FoxO1 antibody revealed that upon Cre mediated excision of the stop cassette FoxO1-ADA was expressed already at E13.5 (Fig. 24C). Western blot analysis of E16.5 epidermis showed an approximately 5 fold higher expression of FoxO1-ADA compared to endogenous FoxO1 (Fig. 24D). The hypoplastic phenotype of FoxO1-ADA mice became first apparent at E14.5 and was sustained throughout epidermal development and in newborn mice, thus indicating that epidermal overexpression of FoxO1-ADA resulted in a major defect in epidermal stratification (Fig. 24B&E). This phenotype is highly similar to the phenotype of p63$^{-/-}$ mice (Yang et al., 1999; Mills et al., 1999).

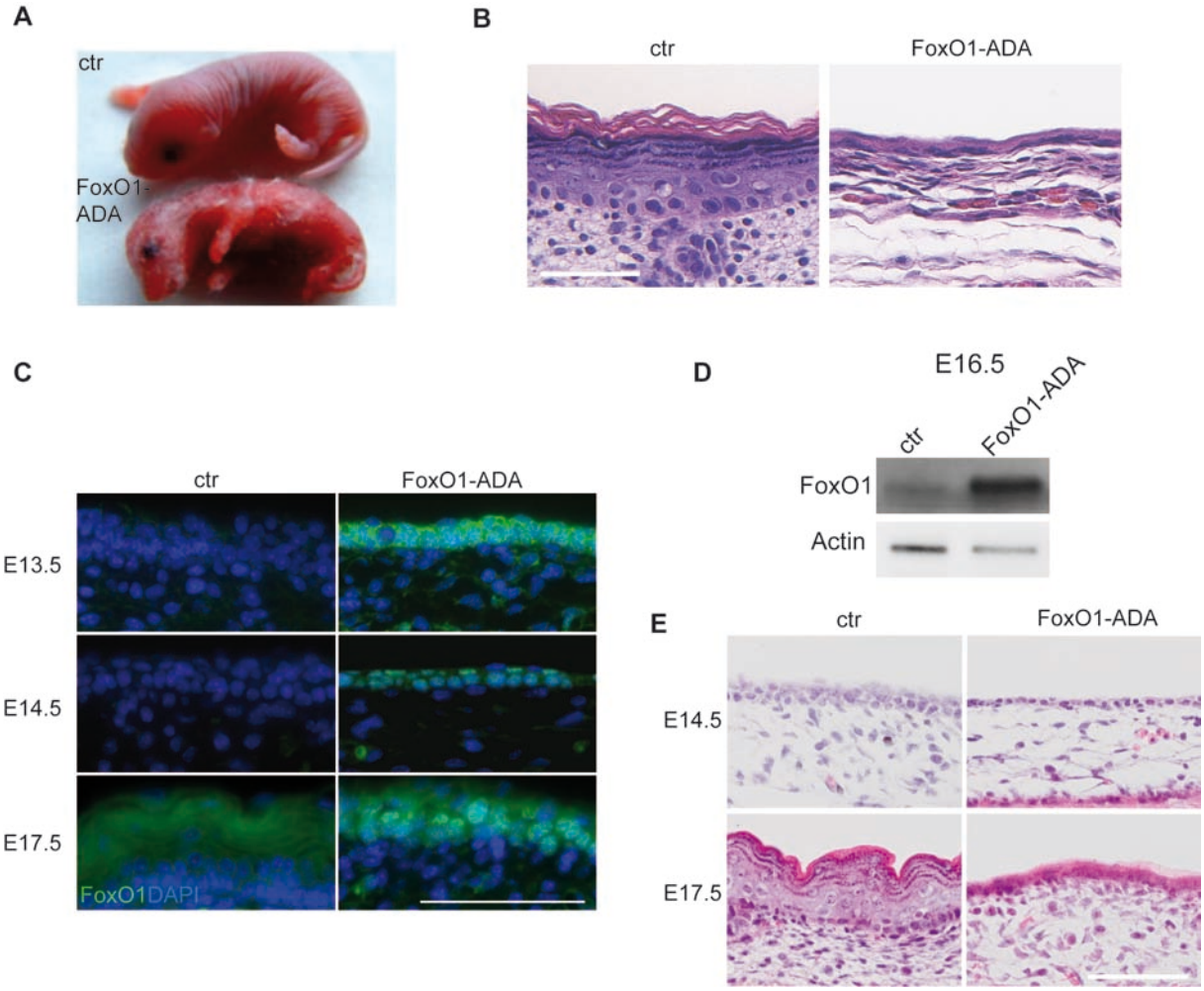


Figure 24: FoxO1-ADA mice show striking epidermal phenotype with hypomorphic epidermis. (A) Macroscopic appearance of control and FoxO1-ADA newborn mice. (B) H&E staining of paraffin sections of newborn control and FoxO1-ADA skin. Scale bar is 50 μm. (C) Immunofluorescence analysis of FoxO1 expression (green) in sections from different embryonic stages in control and FoxO1-ADA mice. Nuclei were counterstained with DAPI (blue). Scale bar is 25 μm. (D) Western blot analysis of FoxO1 on protein lysates of E16.5 epidermis from control and FoxO1-ADA mice. (E) H&E staining on paraffin sections of ctr and FoxO1-ADA embryos at E14.5 and E17.5. Scale bar is 50 μm.

2.16. Strongly impaired stratification in FoxO1-ADA mice

To analyze the FoxO1-ADA-induced stratification defect in more detail, we asked whether the of basal and suprabasal markers is impaired during morphogenesis in FoxO1-ADA mice. Immunofluorescence analysis revealed a loss of signal for suprabasal stratification markers in the developing FoxO-1ADA epidermis (Fig. 25). Keratin 14, which marks the basal cell layer of the epidermis, was normally distributed during embryonic development of FoxO1-ADA mice (Fig. 25 upper panel left). In contrast, staining for Keratin 10, a marker that labels suprabasal layers that is first induced upon formation of the spinous layer, was already decreased at E14.5 in FoxO1-ADA epidermis compared to control (Fig. 25 upper panel right). No staining could be observed for, the late stratification marker loricrin at E14.5 in FoxO-1-ADA embryos whereas control epidermis showed patchy positive staining at this time point, indicating formation of the *stratum granulosum* in these E14.5 embryos (Fig. 25 lower panel left). Interestingly, small patches of K10 and loricrin staining were observed at E15.5 in the FoxO1-ADA epidermis but staining for these markers was absent later during development. The expression of Keratin 6, which is a marker of the late periderm, was also reduced and mislocalized upon epidermal FoxO1-ADA expression (Fig. 25 lower panel right), suggesting that FoxO1-ADA expression might interfere with proper periderm formation. These data show that FoxO1-ADA mice fail to initiate a proper stratification program during epidermal morphogenesis resulting in a very strong reduction of suprabasal layers.

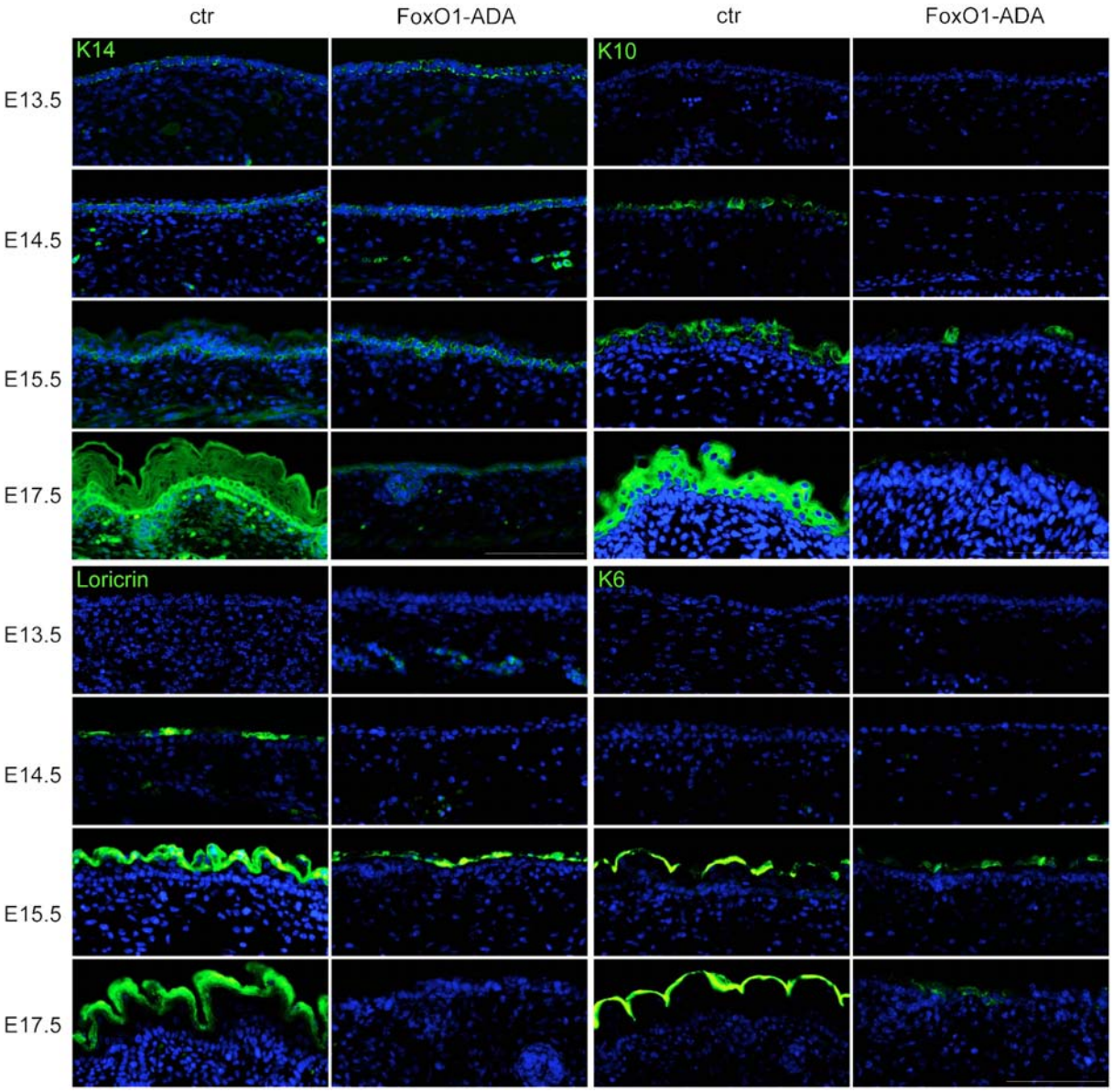


Figure 25: Impaired stratification in FoxO1-ADA embryonic epidermis. Immunofluorescence analysis of stratification markers during epidermal development in control and FoxO1-ADA epidermis. Keratin 14 (K14, green) is a marker of the basal epidermal layer (upper left panel). Keratin 10 (K10, green, upper right panel) and Loricrin (green, lower left panel) are markers of the suprabasal epidermal layers. Keratin 6 (K6, green, lower right panel) is a marker for the periderm. Nuclei were counterstained with DAPI (blue) and the scale bar is 50 μm.

2.17. No increase in apoptosis in FoxO1-ADA mice

FoxOs have been implicated in the regulation of apoptosis (Eijkelenboom and Burgering, 2013) and the loss of suprabasal layers in the FoxO1-ADA mice might thus be explained by increased cell death. However, TUNEL stainings on embryonic sections revealed no increase in apoptosis in the developing epidermis of K14-Cre-FoxO1-ADA mice compared to control, similar to what was observed in dko epidermis (Stachelscheid et al., 2008), and thus likely is not causative of the phenotype.

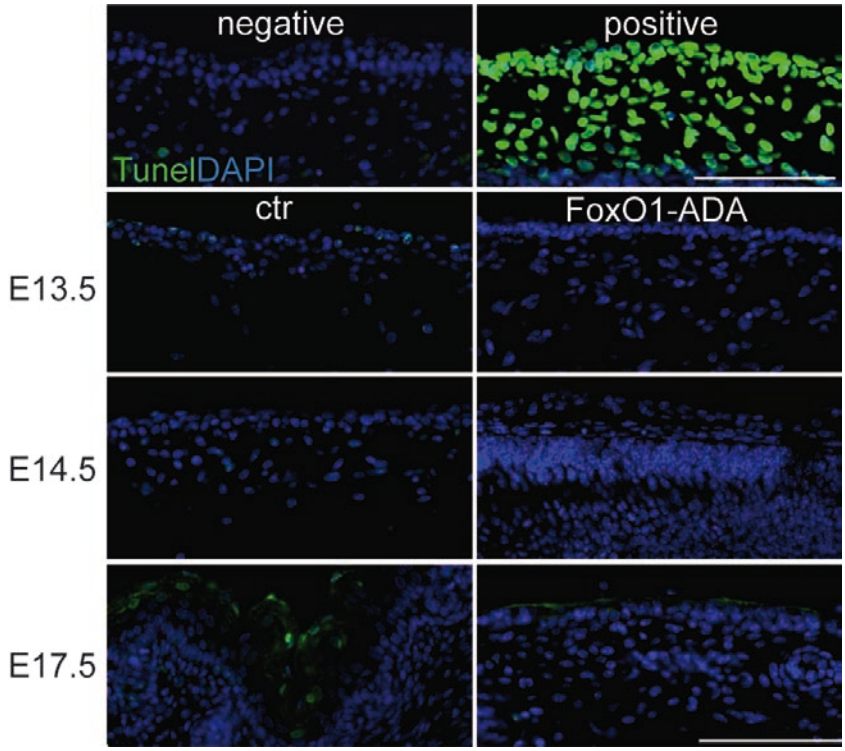


Figure 26: Apoptosis is not increased in FoxO1-ADA epidermis. TUNEL staining analysis (green) of control and FoxO1-ADA expressing epidermis at embryonic days E13.5, E14.5 and E17.5. Nuclei were counterstained with DAPI (blue). Negative indicates non-labeled control and positive indicates a DNAse treated positive control. Scale bar is 50 µm.

2.18. Biased loss of ACD in FoxO1-ADA mice

The phenotype of FoxO1-ADA mice, although much stronger, resembled the epidermal loss of IIS and phenol-copied the $p63^{-/-}$ mice. If the phenotype is indeed caused by similar mechanisms as in these mice one would predict that FoxO1-ADA expression would result in biased loss of ACD. We first quantified the number of cells in anaphase and, similar to the IGF-$1R^{epi-/-}$ and the dko^{epi} epidermis, FoxO1-ADA mice showed a strong reduction of anaphase spindles at E16.5 compared to control (Fig. 27A), thus suggesting an arrest in mitosis, as observed upon epidermal loss of IIS. This reduction in anaphase spindle divisions was even more decreased in the FoxO1-ADA mice compared to the dko^{epi} epidermis and indicates that FoxO is indeed downstream of IIS in the regulation of epidermal morphogenesis. More importantly, analysis of spindle orientation revealed a shift from predominantly SCD to ACD in E16.5 FoxO1-ADA embryos compared to control (Fig. 27B). Again, as shown for the loss of epidermal IIS this shift was driven by a strongly biased loss of ACDs to approximately 10% of control ACDs, whereas the relative number of SCDs was not significantly altered (Fig. 27C). This biased loss of ACD in FoxO1-ADA mice is also similar to the loss of ACD observed in $p63^{-/-}$ mice (Lechler and Fuchs, 2005).

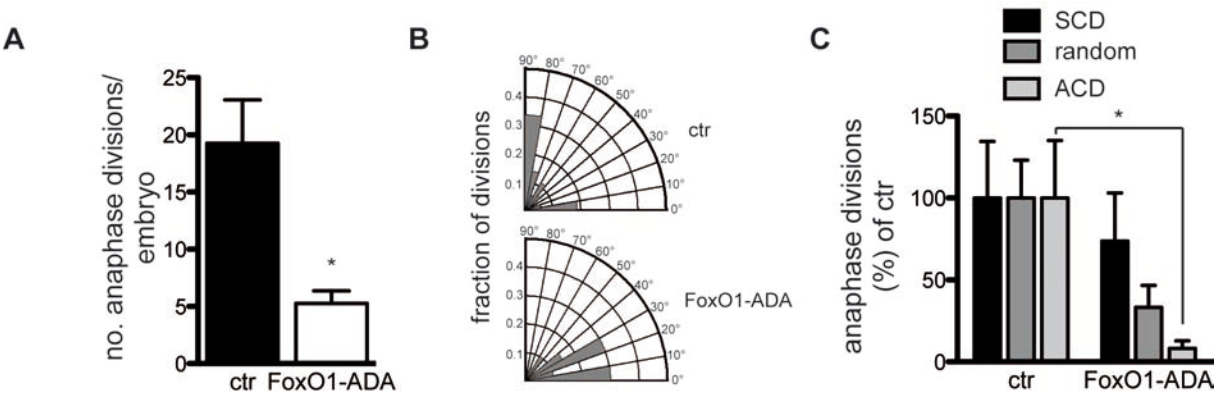


Figure 27: ACD is severely impaired in FoxO1-ADA epidermis. (A) Quantification of anaphase cell divisions in E16.5 control and FoxO1-ADA mice in the basal epidermal layer. Shown is the mean of n=3 embryos/ genotype ± SD. Statistical significance was tested using student's T-test and indicated

as *p<0.05. (B) Analysis of division orientation based on survivin staining of basal epidermal keratinocytes at E16.5 in control and FoxO1-ADA mice. Radial histograms show the relative number of division angles (n=3 of each genotype). (C) Relative comparison of symmetric, random and asymmetric divisions in control (n=70 divisions) and FoxO1-ADA (n=22 divisions) epidermis at E16.5. Angles of divisions were categorized in 0-30° as SCD, 30-60° as random and 60-90° as ACD-Significance was tested separately for each division type using student's T-test and indicated as *p<0.05.

2.19. Altered p63 target gene expression in FoxO1-ADA mice

If FoxO is downstream of IIS signaling in the regulation of epidermal morphogenesis and altered IIS/FoxO activity would impair *in vivo* stratification by inhibiting p63 transcriptional activity, then p63 target genes should also be altered in the FoxO1-ADA epidermis. To investigate this we isolated RNA from E16.5 FoxO1-ADA and control epidermis and performed realtime qPCR analysis for p63 target gene expression. As expected, a range of p63 target genes showed an altered expression in 16.5 FoxO1-ADA epidermis, most of which were also altered in IGF-1R$^{epi-/-}$ epidermis, whereas targets not altered by loss of IIS were again not changed (Fig. 28A). Furthermore, we investigated whether p63 localization was altered in FoxO1-ADA mice. No change in p63 localization or staining intensity was observed in the basal layer of FoxO1-ADA epidermis compared to control, indicating that FoxO1-ADA expression did not alter p63 localization. This is in agreement with the observation that loss of IIS signaling also did not alter p63 RNA or protein expression (see Fig. 14B&D). Together, these data suggest that nuclear FoxO is a crucial regulator of p63 transcriptional activity at E16.5.

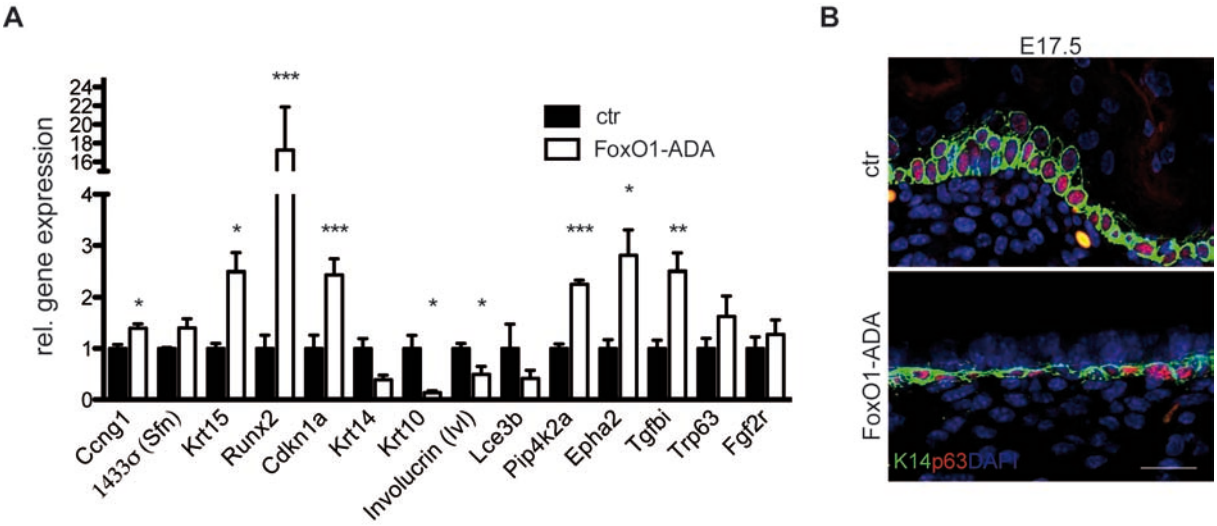


Figure 28: Epidermal FoxO regulates p63 target gene expression. (A) Realtime qPCR analysis of RNA isolated from E16.5 control and FoxO1-ADA epidermis and tested for expression of p63 target genes, which were altered in E16.5 IGF-1R$^{epi-/-}$ mice (see Fig. 14). Shown are n=4 embryos/genotype ± SEM and the control was set to 1. Statistical significance was tested using student's T-test and indicated as *p<0.05, **p<0.01 and ***p<0.001. (B) Immunofluorescence analysis of p63 (red) and Keratin 14 (green) in epidermis of E17.5 FoxO1-ADA mice. Nuclei are counterstained with DAPI (blue). Scale bar is 10 μm.

2.20. Wnt/β-catenin signaling is impaired in FoxO1-ADA mice

Although the stratification phenotype of p63$^{-/-}$ (Yang et al., 1999; Mills et al., 1999) and the FoxO1-ADA mice in the IFE is very similar, epidermal FoxO1-ADA mice did develop hair follicles during morphogenesis , in contrast to p63$^{-/-}$ mice (Fig. 29A). As Wnt/β-catenin is an important regulator of hair follicle initiation (Huelsken et al., 2001), and FoxOs have been shown to regulate β-catenin, we asked whether Wnt/β-catenin target genes were altered by the expression of FoxO1-ADA. To investigate β-catenin targets we isolated RNA from E16.5 control and FoxO1-ADA epidermis and performed realtime qPCR analysis. Interestingly, several β-catenin signaling targets, such as *Axin2*, *Ccnd1* and *Wnt3*, are significantly increased at this developmental

stage (Fig. 29B). This suggests a potential role for nuclear FoxOs in the regulation of hair follicle development.

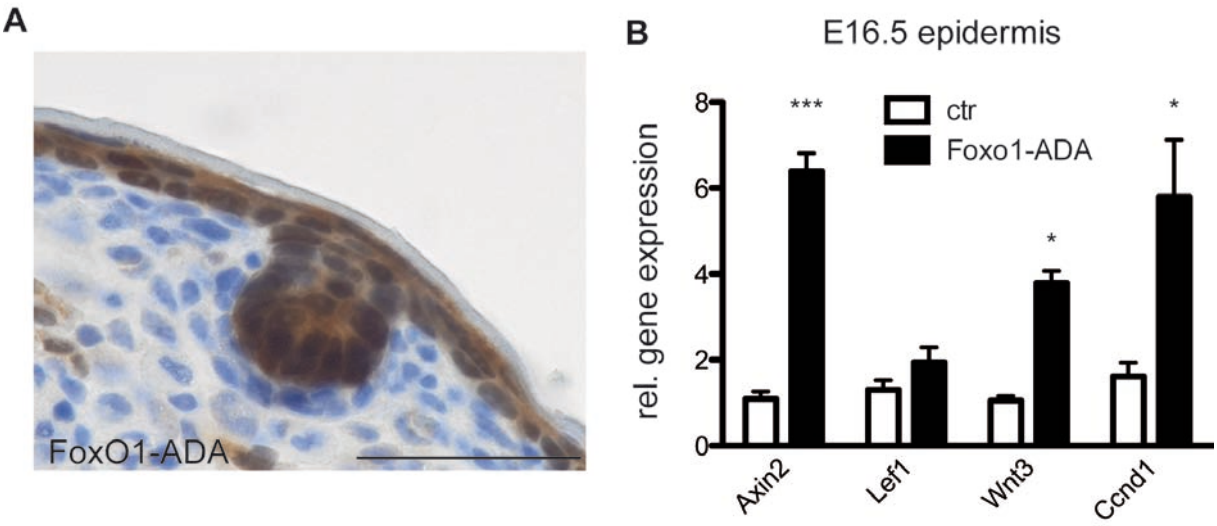


Figure 29: FoxO1-ADA mice develop hair follicles. (A) Histochemical analysis of FoxO1 (brown) expression in E16.5 FoxO1-ADA epidermis. Scale bar is 50 μm. (B) Realtime qPCR analysis of RNA isolated from E16.5 control and FoxO1-ADA epidermis and tested for the expression of β-catenin target genes. Shown are n=3 embryos/genotype ± SD and the control was set to 1. Statistical significance was tested using student's T-test and indicated as *p<0.05 and ***p<0.001.

3. Discussion

This study identified a role for IIS in FoxO-dependent control of p63, a key determinant of epidermal cell fate (McKeon, 2004), to regulate ACD and progression of mitosis during epidermal morphogenesis. We could demonstrate that the loss of IIS leads to a hypomorphic epidermis at E16.5. This was accompanied by a mitotic arrest, a biased loss of ACDs and alterations in p63 transcriptional activity. Furthermore, we could demonstrate that IIS regulated FoxOs impair p63 activity by the direct binding of p63 at target promoters independent of FoxO-DNA interaction. Moreover, the expression of constitutive nuclear FoxO1 resulted in a hypomorphic IFE similar to the epidermis of $p63^{-/-}$ mice and was due to less proliferation, a loss of ACDs and alterations of p63 target genes. In contrast to $p63^{-/-}$ mice, these FoxO1-ADA mice developed hair follicle plugs, suggesting an additional role for nuclear FoxO in hair follicle initiation. In agreement with the role of Wnt/β-catenin signaling for hair follicle formation, FoxO1-ADA mice showed altered Wnt/β-catenin target gene expression (Huelsken et al., 2001).

3.1. A role for IIS in mitotic progression

In the previous work by Heike Stachelscheid a role for IIS in the control of G2/M or mitotic block was identified. It was found that keratinocytes lacking IIS arrested in this stage. Here, we show that IIS is sufficient to drive cells into cell cycle upon release from the mitotic block. This is intriguing, as IIS has been shown to regulate G1/S transition rather than G2/M or mitotic block in other cells (Stull et al., 2002; Mairet-Coello et al., 2009). How IIS regulates this progression in keratinocytes is not clear. In previous findings the expression of the mitotic regulators, such as polo-like kinase 1 (PLK1) and Aurora B, were decreased in dko^{epi} keratinocytes. This could be a reason for the mitotic arrest and the loss of anaphase cell divisions observed in these

mice. In addition, mice that lack epidermal Aurora A, a related Aurora kinase, fail to develop a stratified epidermis similar to dko^{epi} mice (Torchia et al., 2013). This hypoplastic epidermal phenotype was accompanied by an increased number of phospho-Histone 3 positive keratinocytes in the basal layer, which was not observed in the dko^{epi} mice. As Aurora B is required for Histone 3 phosphorylation at Ser10 (Hsu et al., 2000), it suggests that histone 3 phosphorylation during epidermal development in dko^{epi} mice might be disturbed. This could impair chromosome segregation and result in a G2/M arrest. Aurora A has been identified as a downstream target of the IIS target Akt kinase and in response to Akt inhibition Aurora A expression decreases resulting in disorganized spindles, defects in centrosome separation and finally in G2/M arrest (Liu et al., 2008). Furthermore, it has been shown that the activation of Akt, via overexpression of a constitutively active form, could overcome DNA-damage induced G2/M cell cycle checkpoint arrest (Kandel et al., 2002). These results indicate that the PI3K/Akt pathway plays a role in the regulation of the G2/M transition and perhaps Aurora kinases are putative targets of IIS in keratinocytes.

FoxO transcription factors are downstream targets of IIS/Akt, which have been shown to control G1/S cell cycle progression by the activation of CDK inhibitors, such as *Cdkn1a* (p21) or *Cdkn1b* (p27) (Ho et al., 2008). Interestingly, FoxOs are also implicated in G2/M transition regulation. Thus, overexpression of FoxO1 decreased the expression of genes essential for G2/M transition, such as CDK2, cyclin B1 and B2 (Takano et al., 2007). Cyclin G2, an unconventional cyclin that facilitates G2/M arrest by inhibiting cyclin B/CDK1 complex formation, has been identified as a direct target of FoxOs (Martinez-Gac et al., 2004). In response to DNA damage, FoxO3a promotes the expression of *Gadd45a* to mediate G2/M cell cycle arrest and trigger DNA repair (Tran et al., 2002), which blocks cyclin B/CDK2 activation (Zhan et al.,

1999). Although this was demonstrated in other cell types, it suggests that the IIS/Akt/FoxO axis could regulate G2/M transition in keratinocytes. To test this, experiments that investigate the expression of these FoxO regulated genes in FoxO1-ADA mice and dko^{epi} mice would be important.

Another potential regulator of G2/M or mitotic block regulation downstream of IR/IGF-1R is the p38 MAPK pathway. P38 is able to induce a G2/M checkpoint arrest either by the activation and stabilization of p53 (Bulavin et al., 1999) or by the phosphorylation and inhibition of the phosphatase Cdc25B (Bulavin et al., 2001). Cdc25B dephosphorylates CDK2 and activates the cyclin B/CDK2 complex driving the progression of the cell cycle. This would be a potential pathway by which G2/M transition is regulated in response to IIS. In line with this, p38 phosphorylation was increased in the dko^{epi} newborn epidermis.

3.2. The role of IIS/FoxO in the regulation of SCD and ACD

Stratification is a crucial process to induce and maintain the multiple epidermal layers. During this process the suprabasal layers are initiated at around E14.5, which is accompanied by the commitment to terminal differentiation of cells in the most outer layer (Smart, 1970). One model that tries to connect basal cell proliferation and suprabasal differentiation suggests the contribution of SCDs and ACDs during stratification (Smart, 1970; Lechler and Fuchs, 2005). In this study we suggest that IIS via FoxO regulation controls cell division orientation as the loss of IIS results in less asymmetrically dividing cells (ACDs).

The ratio of SCD to ACD is determined by specific polarity signals in the epidermis. Lechler and Fuchs (2005) suggested that this polarity regulation is regulated by the transcription factor p63 (Lechler and Fuchs, 2005). In this study we identified p63 as a target of IIS, but were unfortunately not able to elucidate the role of IIS-p63 in the

regulation of polarity regulators, such as Par3, NuMA and LGN. Previous data showed that the expression of Insc and the recruitment of NuMA to the apical cell cortex is necessary for ACDs in the epidermis (Poulson and Lechler, 2012) and that $p63^{-/-}$ did not alter the expression or localization of these proteins, suggesting that p63 cannot directly control these polarity regulators. In addition, the knockdown of either LGN or NuMA during stratification initiation results in a strong decrease of ACDs and increased SCDs in the basal epidermal layer and this switch affects Notch signaling (Williams et al., 2011). Given these findings, it would be interesting to investigate IIS deficient epidermis for alterations in the Notch signaling pathway. In contrast to the phenotype of the dko^{epi}/FoxO1-ADA mice, which showed a severe decrease in ACD, mice with the epidermis specific deletion of the Par-complex member aPKCλ produced more ACDs in different hair follicle compartments (Niessen et al., 2013). If there is a relation between IIS and aPKC, it would suggest that loss of IIS leads to alterations in aPKC expression or localization.

Although the deletion of p63 did not alter the expression of Insc and NuMA, several polarity regulators, such as Par3 and NuMA, have putative p63 binding sites in their promoters, which were identified by whole genome ChIP-seq analysis for p63 binding sites in human keratinocytes (Kouwenhoven et al., 2010). This suggests, at least in human keratinocytes, that p63 could regulate to some extend the expression of these regulators and thereby contribute to the SCD/ACD regulation.

Alternatively, it was shown that components of the IIS cascade contribute to ACD. Thus, Akt, the downstream target of IIS, has been implicated in ACD regulation in cancer cells and it has been demonstrated that Akt is asymmetrically suppressed in one of the daughter cells (Dey-Guha et al., 2011). Interestingly, mice with a combined loss of Akt1 and Akt2 have less suprabasal layers, which is similar to the epidermal loss of IR and IGF-1R phenotype (Peng et al., 2003). Therefore, it would

be interesting to investigate these mice for SCDs and ACDs. Moreover, the small GTPase Rac, which previously was shown to mediate IIS regulation on epidermal morphogenesis (Stachelscheid et al., 2008), has also been implicated in the control of ACD in drosophila germline stem cells (Lu et al., 2012).

However, how IIS and p63 regulate cell polarity and SCD/ACD in the mammalian epidermis is still poorly understood and will need further investigations.

3.3. The role of IIS regulation of p63 in epidermal development

The transcription factor p63 is crucial for the formation of stratifying epithelia such as the epidermis. Here we unravel a new mechanism that regulates p63 activity. So far, the upstream regulators of p63 were largely unknown and only Bmp signaling in zebrafish has been shown to regulate p63 activity (Bakkers et al., 2002). In this study was shown that p63 is an ectoderm-specific direct transcriptional target of Bmp signaling required for non-neural cell development and thereby initiates epidermal development (Bakkers et al., 2002). Here, we identify IIS as an upstream regulatory pathway for p63. We could show that in mice lacking epidermal IIS the regulation of a subset of p63 targets, such as 14-3-3σ and Runx2, was altered at E16.5, suggesting the loss of p63 function. Later, in newborn mice this resulted in a decreased expression of p63 itself in dko^{epi} mice, probably caused by a self-regulatory loop on a specific enhancer with conserved p63 binding sites (Antonini et al., 2006). Interestingly, the alteration of 14-3-3σ is reflected in the phenotype of 14-3-3σ transgenic mice. 14-3-3σ expression is up-regulated in suprabasal epidermal layers, suggesting a role for this protein in the control of cell cycle exit terminally differentiating keratinocytes (Dellambra et al., 2000). The epidermis specific overexpression of 14-3-3σ resulted in a hypomorphic epidermal phenotype (Cianfarani et al., 2011). Moreover, these mice have less suprabasal layers and

keratinocytes have a reduced proliferative potential (Cianfarani et al., 2011), which is similar to IGF-1R$^{epi-/-}$ and dko^{epi} mice. Runx2 null mice showed a slightly but significant thinner epidermis and decreased proliferation based on Ki67 staining (Glotzer et al., 2008). This is in contrast to our observation that Runx2 is up-regulated in IR/IGF-1R deficient epidermis, which might be explained by differential regulation of the *Runx2* gene. As shown in osteogenesis, FoxO3a in complex with Sirt1 can regulate Runx2 (Tseng et al., 2011).

Another important observation in IGF-1R$^{epi-/-}$ and dko^{epi} mice is the alteration of genes of the EDC. This gene cluster is largely regulated on epigenetic level by different chromatin remodelers (Botchkarev et al., 2012). P63 has been involved in the regulation of these chromatin organizers. Thus, it was demonstrated that p63 regulates the genome organizer Satb1, which is important for higher order chromatin regulation (Fessing et al., 2011). This is in line with our observation that IIS perturbes p63 function, which could lead to altered regulation of Satb1 resulting in the increased expression of EDC genes, such as *Sprr2f* and *Lce3b* (Fig. 14C). But also other chromatin regulating proteins could be responsible for the alteration of this locus. One possible candidate downstream of IIS is FoxO. It was shown that FoxO1 is able to disrupt the histone-DNA contacts leading to the de-compaction of nucleosomal arrays and that the C- as well as the N-terminal regions of FoxO1 can interact with core histones H3 and H4 (Hatta and Cirillo, 2007). This would mean that FoxO-p63 regulation is not necessary for the EDC opening and gene expression. Another EDC regulator is c-MYC and its related regulatory chromatin organizer, such as Setd8 and Sin3a. For example, the histone methyltransferase Setd8 is required for epidermal homeostasis. Thus, the conditional deletion of Setd8 in the epidermis resulted in the loss of p63 expression, most likely by epigenetic reorganizations, and the increase in p53 expression (Driskell et al., 2012). These mice, similar to dko^{epi}

mice, have a severe hypomorphic epidermal phenotype and fail to develop a stratified epidermis (Driskell et al., 2012). The transcriptional regulator Sin3a functions as co-repressor of c-MYC. It was demonstrated the loss of Sin3a results in increased c-MYC binding to the EDC genes and thereby a transcriptional activation of these genes (Nascimento et al., 2011). If the EDC is regulated in a FoxO-p63 dependent manner, need further investigation. Therefore, ChIP-seq investigations of p63 and FoxO could elucidate the regulation of these genes in the IIS deficient epidermis and how this regulation contributes to the barrier defect of dko^{epi} mice.

The transcription factor p63 is a member of the p53 family of transcription factors and is able to bind similar target promoters as p53 (Truong et al., 2006). Interestingly, the investigation by Heike Stachelscheid showed that the expression of p53 is increased in newborn $IGF\text{-}1R^{epi-/-}$ and dko^{epi} epidermis. This could explain changes in p63 target gene expression and contribute to the phenotype. However, unpublished data in the lab indicate that loss of p53 did not rescue the hypomorphic epidermal phenotype of $IGF\text{-}1^{epi-/-}$ mice (not shown), indicating that increased p53 action is not responsible developmental phenotype of IIS deficient epidermis.

In general, p63 is a crucial regulator of epidermal development and homeostasis, which regulates proliferation and differentiation. Here, we show a novel regulator of p63 activity. But overall, the precise function how p63 mediates these processes and how it contributes to the regulation of SCD and ACD in response to IIS needs to be further investigated.

3.4. The interaction of FoxO and p63

IP experiments identified FoxO transcription factors as direct interaction partners of p63. This interaction alters p63 target gene expression and is independent of FoxO-

DNA binding. P63 is highly similar to the p53 tumor suppressor and has a key function in ectodermal development and homeostasis.

It has been demonstrated that FoxO3a is able to interact with p53 to regulate Sirt1 expression in response to changed nutrient availability (Nemoto et al., 2004). This Sirt1 regulation was independent of FoxO3a-DNA binding and resulted in the reversion of p53 activity on Sirt1. The FKH-DNA binding domain and the C-terminal CR3 domain of FoxO3a mediate this interaction, which embrace p53 (Wang et al., 2008). Interestingly, the FoxO3a binding sites are found in the DBD of p53, which has the highest conservation, with over 60% sequence identity, in the p53 family of transcription factors (Vanbokhoven et al., 2011). This together with the high conservation among FoxOs in the FKH-domain and CR3 domain (Wang et al., 2008) suggest the possible interaction of FoxOs with other p53 family proteins. Thus, for p63 we could demonstrate the interaction with FoxO1. In addition, the DBD only FoxO1 mutant (FoxO1-DN) was able to alter p63 function in heterologous CHO cell systems confirming the importance of this domain for the p53/p63-FoxO interaction. Moreover, FoxOs interact with a number of additional transcriptional regulators, which are important for IFE morphogenesis and homeostasis. One example is β-catenin. Thus, it was shown that FoxO3a as well as FoxO4 compete with TCF transcription factors for β-catenin binding (Almeida et al., 2007; Hoogeboom et al., 2008). This resulted in β-catenin mediated increase in FoxO target gene transcription of e.g. C*atalase* and *Gadd45* and vice versa altered the expression of TCF regulated target genes, such as *Axin2*, in response to increased oxidative stress (Almeida et al., 2007). Furthermore, it was recently suggested that autocrine Wnt/β-catenin signaling regulates IFE stem cell self-renewal and thereby maintains the IFE homeostasis (Lim et al., 2013; Choi et al., 2013). If nuclear FoxO interferes with Wnt/β-catenin

regulation in keratinocytes one could speculate that these IFE stem cells are altered or even lost.

Another interaction partner of FoxOs, which has been shown to regulate IFE homeostasis, is c-MYC (Jensen et al., 2011). MYC is predominantly expressed in the basal layer of the IFE and promotes differentiation (Watt et al., 2008). In addition to p63, FoxO could also interact with MYC and thereby impair the development of differentiated suprabasal layers.

Nevertheless, there are multiple possibilities how FoxO might interfere with epidermal morphogenesis independent of FoxO-DNA binding, but how these are involved in the regulation of ACD and stratification remains to be analyzed. Moreover, the DNA binding independent function of FoxOs to regulate transcriptional programs seems to be an important mode of FoxO activity.

3.5. The role of FoxO during hair follicle development

In contrast to $p63^{-/-}$ mice, FoxO1-ADA mice develop hair follicle, which suggests that the abundance of active FoxO in the nucleus does not alter the initiation of hair follicles. As FoxO1-ADA is already expressed at E13.5 (Fig. 24C), a time-point when no hair follicle plugs can be observed in control mice, one could speculate that nuclear FoxO is crucial or at least does not compromise hair follicle development.

This observation is further emphasized by alterations in Wnt/β-catenin signaling in the epidermis of E16.5 FoxO1-ADA mice (Fig. 29B). Wnt/β-catenin is a crucial regulator of hair follicle initiation and the deletion of epidermal β-catenin results in mice, which do not develop proper hair follicles (Huelsken et al., 2001). FoxOs are able to compete with TCF for β-catenin binding. This leads to a mutual inhibition resulting in differential regulation of FoxO and Wnt target genes (Almeida et al., 2007; Hoogeboom et al., 2008). If FoxO is important for hair follicle formation or

homeostasis, it suggests a balanced regulation of FoxO and Wnt-signaling targets in hair follicles during epidermal development and homeostasis. In line with this hypothesis, investigations of mice that express a nuclear mutant of Akt show alterations of hair follicle cycling (Segrelles et al., 2008), which is accompanied by hyper-phosphorylation of FoxO3 in tumor-derived keratinocytes of these mice indicating FoxO inactivation (Segrelles et al., 2006). In addition, another regulator downstream of IR/IGF-1R and upstream of FoxO, the protein phosphatase PTEN, has been demonstrated to control hair follicle cycling. Mice with epidermis specific deletion of PTEN showed epidermal hyperplasia and accelerated hair follicle morphogenesis. Moreover, in these mice a higher density of hair follicles and less IFE was observed (Suzuki et al., 2003). These data suggest that mediators of IIS are important for hair follicle morphogenesis and homeostasis and that FoxOs could contribute to this regulation. In contrast to these observations, the hair follicles in IGF-1R$^{epi-/-}$ mice looked normal and alterations in the hair cycling were transiently and rarely seen. This could be due to other upstream regulators of FoxOs and might be related to TGF-β/Smad signaling. Thus, it was shown that TGF-β regulated Smad proteins can form complexes with FoxO transcription factors (Seoane et al., 2004) and both together can cooperatively regulate target genes in keratinocytes, such as *Ovol1* and *Gadd45a* (Gomis et al., 2006). In addition to TGF-β, Smads are regulated by the Bmp receptor signaling cascade and this interplay is important for hair follicle stem cell activation (Oshimori and Fuchs, 2012).

As FoxOs are at the crossroads of multiple upstream signaling pathways, which have been shown to regulate hair follicle development and homeostasis, such as Wnt/β-catenin signaling and TGF-β1/Bmp signaling, it is possible that they are crucial contributors to hair follicle regulation. However, if FoxO is important for these

processes needs to be tested by the conditional deletion of the different FoxO isoforms.

3.6. Model

Our data revealed a novel mechanism by which IIS regulates the onset of stratification and normal epidermal development (Fig. 30). When insulin/IGF-1 bind to its receptors IIS leads to Akt phosphorylation and subsequently Akt translocates to the nucleus and phosphorylates FoxOs. Thereby FoxOs are excluded from the nucleus and are unable to bind p63 resulting in normal p63 transcriptional activity. These target genes are important for keratinocyte proliferative potential, ACD and stratification. Thus, the keratinocytes of the basal layer can divide asymmetrically and give rise to more suprabasal layers and finally form a barrier. When IIS is impaired, more FoxO remains in the nucleus and alters p63 function. This leads to altered p63 target gene expression and a loss of proliferative potential and less ACDs resulting in a hypomorphic epidermis. Furthermore, the constitutive expression of nuclear FoxO1-ADA impaired the p63 function resulting in an epidermal phenotype similar to $p63^{-/-}$ mice and the complete loss of ACDs and stratification.

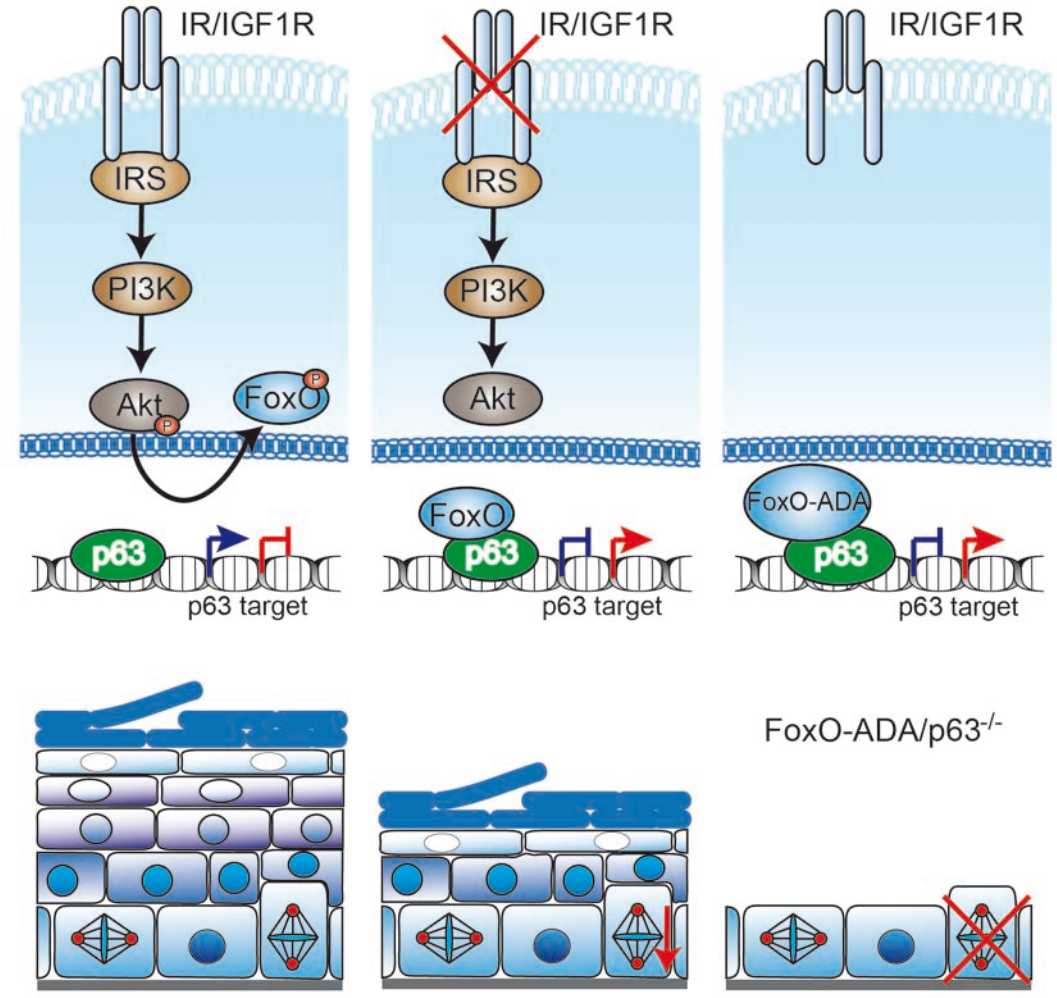


Figure 30: **Model of IIS mediated regulation of FoxO and p63 during epidermal morphogenesis.** IRS- insulin receptor substrate, PI3K- phosphoinositide-3 kinase, Akt- protein kinase B, FoxO- forkhead box O factor

3.7. Future prospects

During epidermal morphogenesis p63 is an important regulator of proliferative potential and stratification and we could show that IIS is a crucial upstream regulator to inhibit FoxO mediated p63 impairment. However, we were not able to rescue epidermal stratification *in vivo.* Thus, it would be important to analyze IGF-1R$^{epi-/-}$ mice with a conditional ablation of FoxOs to conclude about the biological importance of this FoxO driven mechanism during epidermal stratification. Furthermore, it would be important to investigate potential targets of IIS/FoxO/p63 regulation to identify important regulators of ACD and epidermal stratification.

In addition, it is not clear how IIS regulates the spindle block in keratinocytes. A potential interesting downstream target of IIS could be the p38 MAPK pathway, which has been implicated in the regulation of similar processes (Bulavin et al., 2001).

Finally, FoxO is a known regulator of haemapoetic and neuronal stem cells, therefore it would be interesting to investigate the role of FoxOs in distinct IFE and hair follicle stem cell populations and as initial analysis suggest FoxO might be crucial for hair follicle morphogenesis.

4. Material and Methods

4.1. Mice

4.1.1. Epidermis specific IR and IGF-1R knockout mice

The epidermis specific deletions of IR and IGF-1R have been described (Stachelscheid et al., 2008). Briefly, IGF-1R floxed mice were generated using a targeting vector spanning a region from intron 2 to intron 3 of the IGF-1R gene flanked by loxP sites. Cre mediated recombination leads to the excision of exon3 and a frame shift resulting in a stop codon in exon4. Genotyping for IGF-1R$^{fl/fl}$ was performed by PCR on DNA from tail cuts using the following primers: 5'-TCC CTC AGG CTT CAT CCG CAA-3' (fwd) and 5'-CTT CAG CTT TGC AGG TGC ACG-3' (rev). In the IR mice exon 4 is floxed and will be excised upon Cre recombination. For genotyping following primers were used: 5'-GAT GTG CAC CCC ATG TCG-3' (fwd); 5'-CTG AAT AGC TGA GAC CAC AG-3' (rev). IGF-1R$^{fl/fl}$ mice and IR$^{fl/fl}$ mice were crossed with the Keratin 14- Cre mice (Hafner et al., 2004) to generate epidermis specific deletion.

4.1.2. FoxO1-DN and FoxO1-ADA mice

For epidermis specific expression of FoxO1 mutants, mice carrying a CAGG promoter, a loxP-flanked stop cassette followed by the cDNA of constitutively nuclear FoxO1 (FoxO1-ADA) or dominant negative FoxO1 (FoxO1-DN) in the Rosa26 locus were generated (Belgardt et al., 2008; Stohr et al., 2011). For genotyping the following primer sets were used: 5'- GAT ATG GAA TAC TGG GCT CTT-3' (rev); 5'-AAA GTC GCT CTG AGT TGT TAT C-3' (fwd); 5'-TGT CGC AAA TTA ACT GTG AAT C-3' (rev). To conditionally knock-in these mutants specifically in the epidermis, FoxO1 mutant mice were crossed with the Keratin 14 Cre mice (Hafner et al., 2004).

FoxO1-DN mice were additionally crossed with the IGF-1R$^{fl/fl}$ mice to obtain K14-Cre; IGF-1R$^{fl/fl}$; FoxO1-DN mice.

4.1.3. FoxO1-GFP mice

To conditionally express GFP tagged FoxO1, mice with a floxed stop cassette followed by the cDNA of human GFP-tagged FoxO1 at the Rosa26 locus (Fukuda et al., 2008) were crossed with K14-Cre mice (Hafner et al., 2004). For the investigation of the subcellular localization of FoxO1-GFP, these mice were additionally crossed with IGF-1R$^{fl/fl}$ mice to obtain K14-Cre; IGF-1R$^{fl/fl}$; FoxO1-GFP mice. For genotyping of the FoxO1-GFP construct the following primer sets were used: 5'-GGA GGG GAG TGT TGC AAT ACC T-3' (fwd), 5'-GCG GGA GAA ATG GAT ATG AAG T-3' (rev); 5'-CCG GAT CCA CTA GTT CTA GAG C-3' (rev).

4.1.4. Isolation of epidermis of newborn mice

The epidermis of newborn mice was separated from the dermis by floating the whole dissected skin on buffer containing 0.5 M ammonium thiocyanate (NH_4SCN) diluted in phosphate buffer (0.1 M Na_2HPO_4, pH 6.8) for 20 min on ice. After stripping, the epidermis was either snap frozen in liquid nitrogen or immediately processed for RNA isolation or protein extraction.

4.1.5. Isolation of epidermis of E16.5 embryos

Epidermis of embryos was separated using dispase digestion. Therefore the dissected skin was incubated for 2 to 4 hours in mouse keratinocyte medium supplemented with Dispase II (Sigma) at 4°C (500mg/ml diluted in 10 ml medium). After incubation the epidermis was split, briefly washed in PBS and snap frozen in liquid nitrogen or directly processed.

4.2. Immunohistochemistry

4.2.1. Paraffin embedded skin sections

For histology embryos or mouse skin were fixed in 4% PFA and embedded in paraffin. 5 µm paraffin sections were deparaffinized and stained with Haematoxylin & Eosin (H&E) and imaged with an Olympus BX51 microscope. For immunofluorescence paraffin sections were deparaffinized, antigens were retrieved with buffer A, UG or AG (EMS) by high pressure cooking and sections were blocked in PBS containing 5% normal goat serum and 0.1% Triton X-100. Slides were incubated with primary antibody diluted in IF buffer (10% normal goat serum, PBS, 0,1% Triton X) followed by washing and incubation with the appropriate secondary antibodies coupled to Alexa 488, Alexa 594 or Cy3. Nuclei were counterstained with DAPI (Sigma) and sections examined using Olympus IX81 fluorescence or Olympus FV1000 confocal microscopes. Primary antibodies are listed in Table 1.

4.2.2. Division axis orientation determination

To analyze the angle of divisions, the axis of divisions in E16.5 embryos was determined in anaphase/telophase cells by using survivin staining as a marker for spindle orientation, described by Williams et al. 2011 (Williams et al., 2011). The angle of division was determined by measuring the angle of the plane transecting two daughter cells, marked by survivin, relative to the plane of the basement membrane. The angles of divisions were quantified and angle orientation was plotted with Oriana 4 (KCS). The different divisions were then categorized with asymmetric divisions having an angle of 60-90 degrees, random 30-60 degrees and symmetric 0 and 30 degrees. Each of the total number of asymmetric, random or symmetric divisions of the control were then set to 100% to compare the relative loss within each of the division categories to either knockouts or transgene.

4.3. Protein biochemistry and molecular biology

4.3.1. Protein extraction from epidermal splits and keratinocytes

Primary keratinocytes or epidermis was lysed, after washing with PBS, using stringent SDS Lysis Buffer (20mM Tris pH 7.9, 200 mM NaCl, 1% SDS) supplemented with phosphatase inhibitors, mammalian protease inhibitor cocktail (Sigma-Aldrich) and 2 mM PMSF (Sigma-Aldrich) to prevent protein degradation by phosphatases and proteases. For cellular fractionation of primary keratinocytes detergent-free lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) was used and nuclei were extracted after sequential washes with SDS lysis buffer.

Newborn epidermis was lysed by homogenizing the frozen tissue with a mixer mill for 3 min at 30 Hz. Embryonic epidermis was lysed by sequential tissue disruption using syringes with decreasing tip diameter. Cells were washed with PBS twice before the lysis buffer was added and then dissociated from the dish using a cell scraper. Subsequently, the lysates were transferred into reaction tubes and centrifuged for 10 min at 4°C and 13000 rpm. Supernatants were transferred into a new reaction tubes.

Protein concentration was determined by the modified Lowry method (BioRad) according to manufacturers protocol.

4.3.2. SDS-Polyacrylamid-gelelectrophoresis (SDS-PAGE) and immunoblot

For SDS-PAGE protein lysates were dissolved in Laemmli buffer and incubated for 10 min at 95°C. Equal amounts of total protein lysate were loaded on precast 4-12% gels (Novex, Life Technologies) and separated at 40mA per gel.

After running the gels were transferred to PVDF membrane by semidry blotting or tank-blot. Membranes were blocked with 5% of western blot blocking solution (Roche) in TBS-T (0.1% Tween 20, 20 mM Tris pH 7.5, 137 mM NaCl) for 1 hour and

subsequently incubated with the primary antibody diluted in blocking solution over night at 4°C. After washing in TBS-T, the membranes were incubated with the appropriate horseradish peroxidase coupled secondary antibody. Protein bands were detected by chemiluminescence on a luminescence detector (Biometra) using SuperSignal West Pico or SuperSignal West Femto Kit (Thermo Fisher Scientific).

4.3.3. Co-immunoprecipitation

For Co-immuno-precipitations, transiently transfected CHO cells were lysed using HEPES buffer (50 mM HEPES-KOH pH 7.9, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.25% Triton X-100, 1% NP-40) for 20 min at 4°C. Then the nuclei were disrupted by mild sonication and the lysate was pre-cleared using PBS washed Protein A/G agarose beads (Roche) for 1 hour at 4°C. After overnight antibody-incubation at 4°C antibody/protein complexes were precipitated with Protein A/G beads followed by sequential washing in IP buffer (150 mM NaCl, 20 mM Tris pH 8, 2 mM EDTA). The immune-precipitated samples were analyzed by western blot.

4.3.4. RNA extraction

Gene expression was analyzed using quantitative real time RT-PCR. RNA was extracted from keratinocytes and epidermis using Trizol (Invitrogen) followed by ethanol precipitation and the RNeasy Minikit (Qiagen) according to manufacturers protocol.

4.3.5. cDNA synthesis and realtime qPCR analysis

RNA was reversely transcribed with Quantitect Reverse Transcriptase (Qiagen) to obtain cDNA according to manufacturers instructions. CDNA was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems) and PCR was performed

on an ABI StepONE Plus machine. Samples were compared by calculation of the comparative cycle threshold (ΔΔCt) with data normalized relative to 18S and Hprt1. Probes for target genes were ordered from TaqMan Assay-on-Demand Kits (Applied Biosystems). Taqman probes are listed in Table 3.

4.3.6. Global genome expression analysis

For microarray analysis, RNA was isolated from epidermis of control and dko$^{epi-/-}$ newborn mice (N=4 each) and sent to the DNA Sciences Core at the University of Virginia for labeling, amplification and hybridization to the Affymetrix 430-2.0 platform. To analyze global gene expression, significantly regulated gene sets ($p<0.05$) from the different microarrays were analyzed for overlap using VENNY Tool (Oliveros et al., 2007) (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The significance of overlapping and non-overlapping genes was determined using hypergeometric distribution algorithm. Overlapping and non-overlapping gene sets were then annotated using DAVID functional annotation tool (Dennis et al., 2003).

4.3.7. Chromatin-immunoprecipitation followed by qPCR

For ChIP assays approximately 3 *10^6 primary mouse keratinocytes or one total newborn epidermis were cross-linked with 1% formaldehyde. Crosslinking was stopped and cells lysed with SDS lysis buffer. Lysates were extensively sonicated on ice to obtain DNA fragments ranging from 200 to 1000 bp in length. The supernatant was diluted for immunoprecipitation in IP buffer (150 mM NaCl, 20 mM Tris pH 8, 2 mM EDTA) and pre-cleared with protein A beads (Roche). Supernatant was incubated with 4 μg antibody overnight at 4°C and than beads were sequentially washed in IP buffer, high salt buffer (500 mM NaCl, 20 mM Tris pH 8, 2 mM EDTA), LiCl buffer (250 mM LiCl, 10 mM Tris pH 8, 1 mM EDTA, 1% NP-40) and again with

IP buffer (Ferone et al., 2011). Chromatin was eluted with elution buffer (100 mM $NaHCO_3$, 1% SDS), DNA purified by phenol/chloroform precipitation and analyzed by quantitative realtime PCR or protein complexes analyzed by western blot (see 4.3.2.). Realtime PCR was performed using the SYBR Green PCR master mix (Applied Biosystems) in an ABI StepOne light cycler. Primersets for analysis are listed in Table 4.

4.3.8. Site directed mutagenesis

For FoxO1-ADA DNA binding domain (DBD) mutation the QuikChange Sit-Directed Mutagenesis Kit (Stratagene/Agilent) was used. The FoxO1-ADA target plasmid was amplified using the following primers, which contain mutations of the histidin-codon at position 212 of the DBD to arginine: 5'-GGA AGA ATT CAA TTC GCA GGA ATC TGT CCC TTC ACA GC-3' ; 5'-ACC TTC TTA AGT TAA GCG TCC TTA GAC AGG GAA GTG TC-3'. Each primer was used in a separate PCR reaction and amplified according to manufacturers protocol. Transformation was performed using XL1-Supercompetent bacteria (Stratagen/Agilent) according manufacturers instructions. The correct mutagenesis was confirmed by DNA sequencing.

4.4. Cell biology

4.4.1. Isolation and cultivation of primary keratinocytes

Epidermis of newborn pubs was separated from dermis. After overnight dispase II digestion a single cell suspension was obtain by subsequent Trypsin (TrypLE, GIBCO) digestion for 30min. The isolated keratinocytes were plated on collagen type 1 coated dishes in co-culture with a J2 3T3 fibroblast feeder layer and cultured in keratinocyte cell culture medium (DMEM-medium, 10% chelated FCS, 5μg/ml insulin,10 ng/ml epidermal growth factor (EGF), 10^{-10} M cholera toxin, 100 U/ml

penicillin, 10 μg/ml streptomycin, 2mM L-glutamine). Primary mouse keratinocytes were cultured at 32°C, 5% CO_2 and medium was changed every second day. To split the cell, growth medium was removed and keratinocytes were washed with PBS and trypsinized for 10 min at 37°C. Trypsin was inactivated by the addition of keratinocyte medium. The suspension was centrifuged at 850 rpm for 5 min and the supernatant discarded. The cells were resuspended and plated on new pre-coated dishes.

4.4.2. Chinese hamster ovarian cell culture

Chinese hamster ovarian cells were cultivated in Ham's/F-12 (GIBCO) supplemented with 10% FCS and penicillin (100 U/ml) /streptomycin (100 μg/ml streptomycin).

4.4.3. Transfection of keratinocytes and CHO cells and luciferase reporter assays

For overexpression, primary mouse keratinocytes or CHO cells were transfected using Lipofectamine 2000 (Invitrogen) or Lipofectamine (Invitrogen), respectively, at a confluency of 70-90% according to manufacturer's protocol.

For Luciferase assays cells were transfected at 70-90% in 24 well plates. Transfection solution (250 ng of each reporter or expression plasmid, 25 ng Renilla plasmid, 2-3 μg Lipofectamine 2000 (keratinocytes) or Lipofectamine (CHOs) in 100 μl Medium without additives) was prepared and cells washed twice with PBS. 100 μl transfection solution and 200 μl medium (no additives) were added to each well for 5 hours. Cells were washed twice and cultured in normal medium for 24-48 hours post transfection. Afterwards cells were washed twice with PBS, lysed and Luciferase activity measured using the Dual Luciferase Reporter Asay Kit (Promega) according to manufacturers instructions.

4.4.4. RNA interference

To silence FoxO gene expression keratinocytes were transfected with ON-TARGET plus SMARTpool siRNAs (Thermo Fisher Scientific) targeting FoxO1, FoxO3 and FoxO4. Subconfluent primary mouse keratinocytes were transfected using 50nM of each SMARTpool or non-targeting control pool and Lipofectamine2000 Reagent (Invitrogen) according to manufacturer's protocol. Efficient knockdown was observed 48 hours post-transfection by RT-PCR and Western blot analysis.

4.5. Statistical methods

Data were analyzed for statistical significance using two-tailed unpaired Student's t test unless otherwise stated. Relative loss of ACD, random divisons and SCD were tested using One-way ANOVA in Prism 5 (GraphPad). The asterisks shown in graphs correspond to the p-values as stated in the figure legends. The results were presented as the average of at least three independent experiments unless otherwise stated in the legends and arrow bars indicate standard deviation (SD) or standard error of the mean (SEM).

4.6. Antibodies, primer and plasmids

4.6.1. Primary antibodies

Table 1: List of primary antibodies used for IF, WB or IP

Antigen	Species	Dilution	Manufacturer
Actin (C4)	mouse	WB 1:10000	MP Biomedicals
FoxO1 (C29H4)	rabbit	WB 1:1000 IF 1:100	Cell Signaling Technology
FoxO1 (H-128x,sc-11350)	rabbit	IP 4 μg	
FoxO3a (75D8)	rabbit	WB 1:1000	Cell Signaling Technology
FoxO4 (sc-34899)	rabbit	WB 1:500	Santa Cruz Biotechnology
phospho-FoxO1 (S256)	rabbit	WB 1:500	Cell Signaling Technology
Flag M2	mouse	IP 4 μg	Sigma-Aldrich
GFP (ab-13970)	chicken	WB 1:5000	Abcam
GFP (beads)	alpaca	IP 20 μl	Chromotek
IGF-1Rβ (C20, sc-713)	rabbit	WB 1:500	Santa Cruz Biotechnology
Keratin 10	rabbit	IF 1:2000	Covance
Keratin 14	rabbit	IF 1:2000	Covance
Keratin 6	rabbit	IF 1:2000	Covance
Loricrin	rabbit	IF 1:2000	Covance
p63α (H129)	rabbit	WB 1:2000 IF 1:1000	Santa Cruz Biotechnology
p63 (4A4)	mouse	WB 1:2500 IF 1:1500	Santa Cruz Biotechnology
Survivin (71G4B7)	rabbit	IF 1:100	Cell Signaling Technology

4.6.2. Secondary Antibodies

Table 2: List of secondary antibodies used for IF and WB

Antigen	Species	Dilution	Manufacturer
Alexa 488 anti-rabbit	donkey	1:1000	Molecular Probes (Life Tech.)
Alexa 488 anti-mouse	goat	1:1000	Molecular Probes (Life Tech.)
Alexa 594 anti-rabbit	donkey	1:1000	Molecular Probes (Life Tech.)
Cy3 anti-mouse	goat	1:1000	Cell Signaling Technology
HRP anti-rabbit	goat	1:5000	BioRad
HRP anti-mouse	goat	1:5000	BioRad
HRP anti-chicken	rabbit	1:5000	Upstate (Millipore)

4.6.3. Primer

Table 3: List of Taqman probes used for realtime qPCR

Gene Symbol	Probe number
Foxo1	Mm00490672_m1
Foxo3	Mm01185733_m1
Foxo4	Mm00840140_g1
Foxo6	Mm00809934_s1
Hprt	Mm00446986_m1
18s	Mm03928990_g1
Runx2	Mm00501584_s1
Sfn	Mm01180869_s1
Trp63	Mm01144752_m1
Krt15	Mm00492972_m1
Krt14	Mm00516876_m1
Krt10	Mm03009921_m1
Ccng1	Mm00438084_m1
Fgfr2	Mm01269930_m1
Sprr2f	Mm00448855:s1
Sprr2i	Mm007268832_s1
Lce3b	Mm01333146_g1
Cdkn1a	Mm01303209_m1
Tgfbi	Mm00493634_m1
Pip4k2a	Mm00435721_m1
Epha2	Mm00438726_m1
Jag1	Mm00496902_m1

Table 4: List of primers used for ChIP-qPCR assays

Gene Symbol	Sense/ Antisense Primer Sequence
1433sigma (Sfn)	CGATGTGGAGAACCAGAGAG CCAATATGTTTGTTGGACACCT
Cdkn1a	CATGTTCAGCCCTGGAATTG GTAGTTGGGTATCATCAGGTCTC
Runx2	GACTGTCAGGAGCTGGGAAG GGCCATATAGCCTTGCATCA
Fgfr2 (Ferone et al., Embo Mol Med, 2011)	AATGAGCGCGCAAGTTAGAAC GCCGCGCCGAGATGT
negative control (Ferone et al., Embo Mol Med, 2011)	ACTCTGACGGATGGCTCTTCA AGGCAGACTTGTGTGGAGATGA

4.6.4. Plasmids

Table 5: List of plasmids used for protein overexpression and luciferase assays

Plasmid	**Insert**
pcDNA3-FoxO1-ADA	mouse
pcDNA3-FoxO1-DN	mouse
pcDNA3-FoxO1-ADA-ΔDBD	mouse
pCMV5-Flag -FoxO1	mouse
pCMV2-Flag-ΔNp63α	mouse
pCMV2-Flag-ΔNp63α-L514F	mouse
pCMV-Renilla reporter	Renilla
pG13 (p63 reporter)	human
pBDS-2(3x) (p63 reporter)	human
p6xDBE (FoxO reporter)	human
pEGFP	mouse
pcDNA3	no

5. Abbreviations

ACD	asymmetric cell division
ADULT	acro-dermato-ungual-lacrimal-tooth syndrome
AEC	ankyloblepharon-ectodermal defects-cleft lip/palate syndrome
Akt	protein kinase B
AMP	adenosin-mono-phosphate
AMPK	AMP-aktivierte protein kinase
aPKC	atypical protein kinase C
ATM	ataxia telangiectasia mutated protein kinase
ATP	adenosin-tri-phosphate
BAD	Bcl2-associated death promoter
CCND2	cyclin D2
CCNG2	cyclin G2
CDKN1A	cyclin dependent kinase inhibitor 1A
CDKN1b	cyclin dependent kinase inhibitor 1B
CDKN2A	cyclin dependent kinase inhibitor 1A
cDNA	contemplementary DNA
Cre	site specific recombinase
DAPI	4'6-Diamidino-2-phenylindol
DBD	DNA-binding domain
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
ED	ectodermal diysplasia
EDC	epidermal differentiation complex
EEC	ectodermal dysplasia and cleft lip/palate syndrome
EGF	epidermal growth factor
ERK	extracellular-signal regulated kinase
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FoxO	forkhead box O transcription factor
GADD45A	growth arrest and DNA-damade inducible protein
GFP	green fluorescent protein

H&E	hematoxylin & eosin
HRP	horseraddish peroxidase
HSC	hemapoetic stem cells
IF	immunofluorescence
IFE	interfollicular epidermis
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IIS	insulin IGF signaling
IL-1	interleukin 1
Insc	inscutable
IR	insulin receptor
IRS	insulin receptor substrate
JAG1	jagged 1
JNK	c-jun-N-terminal kinase
K1	keratin 1
K10	keratin 10
K14	keratin 14
K15	keratin 15
K5	keratin 5
Klf4	krüppel-like factor 4
LGN	Leu-Gly-Asn repeat-enriched protein
LMS	limb-mammary syndrome
MAPK	mitogen-activated protein kinase
min	minute
ml	milliliter
mRNA	messenger ribonucleic acid
MYC	myelocytomatosis oncogene homolog
NGS	normal goat serum
NSC	neural stem cell
NuMA	nuclear mitotic apparatus protein
PAGE	polyacrylamide gel electrophoresis
Par3	partitioning defect 3
Par6	partitioning defect 6
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDK1	phosphoinositide-dependent protein kinase
PFA	para-formaldehyde
PI3K	phosphatidylinositol 3 kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5triphosphate
PMSF	phenylmethylsulphonylfluorid
PTM	posttranslational modification
qPCR	quantitative PCR
Rac1	Ras-related C3 botulinum toxin substrate
RHS	Rapp-Hodgkin syndrome
RNA	ribonucleic acid
rpm	rounds per minute
SAM	sterile alpha motif
Satb1	special AT-rich sequence-binding protein 1
SCD	symmetric cell division
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
Skp2	S-phase kinase-associated protein
SMAD	mother against decapentaplegic protein
Sod2	superoxid dismutase 2
Sprr	small proline-rich protein
TA	transactivation domain
TBS	tris buffered saline
TCF	T-cell factor protein
TGF	tumor growth factor
TNF	tumor negrosis factor
Wnt	wingless protein
µg	microgramm
µl	microliter

6. References

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8. Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahren nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Carien M. Niessen und Prof. Dr. Jens C. Brüning betreut worden.

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