Role of IGF-1R/IR signaling in epidermal development and morphogenesis

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Abstract

The epidermis protects the organism from external challenges and from dehydration. The establishment, maintenance and restoration of this epithelial barrier are driven by a balance between self-renewal and differentiation of interfollicular epidermal (IFE) progenitor cells. However, the upstream signals that regulate this balance are largely unknown. The major aim of this thesis was to identify and characterize the *in vivo* role of epidermal insulin/IGF signaling, which have been identified as key regulators of growth, metabolism and stem cell behavior in other tissues. Inactivating the insulin receptor ($IR^{-/-}$), the IGF-1 receptor ($IGF\text{-}1R^{-/-}$) or both (dko) specifically in the epidermis resulted in a progressive decrease in the number of suprabasal layers, first obvious at E16.5, although differentiation and apoptosis were unaltered. Instead, the phenotype was directly associated with a strong decrease in proliferative potential *in vitro* and altered IFE progenitor cell behavior *in vivo*. Furthermore, rescue experiments identified the small GTPase Rac1 as a key target of IGF-1R/IR signaling that regulates epidermal morphogenesis *in vivo* and proliferative potential *in vitro*. To address how IR/IGF-1R regulates epidermal morphogenesis, we focused on E16.5 dko embryos, when the phenotype is first obvious. Whereas no change was observed in expression of the proliferation marker Ki67, a reduced number of anaphase spindles and an increased number of metaphase spindles were found in dko mice compared to control, and *in vitro* in cultured keratinocytes. This indicated an arrest in the spindle check point of the cell cycle in the absence of IR/IGF-1R. This was associated with disturbed spindle formation in $IGF\text{-}1R^{-/-}$ metaphase cells and reduced expression of AuroraB, a key regulator of mitosis, as well as other cell cycle regulators, such as p53 and stratifin (14-3-3σ). Interestingly, the reduction in cell divisions upon loss of IR/IGF-R was predominantly on the expense of asymmetric divisions and correlated with reduced p63 expression, a known regulator of this process. Since asymmetric cell divisions have been implicated in regulating epidermal stratification, the results indicate that epidermal Insulin/IGF1 signaling couples cell cycle progression to asymmetric divisions thereby regulating the number of suprabasal layers in the IFE during morphogenesis. In short, the results in this thesis have identified epidermal IR/IGF-1R as key regulators of epidermal morphogenesis, proliferative potential, IFE progenitor cells and asymmetric cell divisions.

Zusammenfassung

Die Epidermis schützt den Organismus vor äußeren Einflüssen und vor Austrocknung. Die Anordnung, Aufrechterhaltung und Wiederherstellung der epidermalen Barriere werden durch einen Ausgleich zwischen Selbsterneuerung und Differenzierung von Vorläuferzellen in der interfollikularen Epidermis gesteuert. Die vorgelagerten Signale, die diesen Ausgleich regulieren, sind noch nicht bekannt. Das Ziel dieser Arbeit war es, die Rolle des Insulin/IGF Signalweg in der Epidermis zu charakterisieren, welcher als Regulator für Wachstum, Stoffwechsel und bei der Stammzellregulierung in anderen Geweben eine große Rolle spielt. Die epidermal-spezifische Inaktivierung des Insulin, des IGF-1 und von beiden Rezeptoren führte zu einer verminderten Anzahl epidermaler, suprabasaler Zellschichten, zuerst sichtbar im embryonalen Stadium E16.5, wobei Differenzierung und Apoptose nicht verändert waren. Stattdessen war der Phänotyp mit einer starken Abnahme des Zellteilungspotentials in Zellen und mit veränderten IFE Vorläuferzellen in der Epidermis verbunden. „Rescue"-Experimente identifizierten die GTPase Rac1 als Ziel von IGF-1R/IR, welcher die epidermale Morphogenese und das Zellteilungspotential regulierte. Um herauszufinden, wie IGF-1R/IR die epidermale Morphogenese reguliert, haben wir uns auf das Embryonenstadium E16.5 konzentriert, der Tag an dem der Phänotyp zuerst auftritt. Während anhand des Markers Ki67 keine Wachstumsveränderungen festgestellt werden konnten, fand sich eine verminderte Anzahl von Anaphasen und eine vermehrte Anzahl von Metaphasen in dko Mäusen und in kultivierten IGF-1R$^{-/-}$ Keratinozyten. Dies deutete auf einen Arrest im Spindel-Kontrollpunkt der Mitose hin. Der Phänotyp war assoziiert mit einer gestörten Spindel Formation in IGF-1R$^{-/-}$ Metaphase Zellen und mit einer verminderten AuroraB Expression, ein wichtiger Regulator in der Mitose. Zugleich waren weitere Zellzyklus Regulatoren wie p53 und Stratifin (14-3-3σ) verändert. Die IGF-1R/IR Inaktivierung beeinträchtigte hauptsächlich die asymmetrische Zellteilung mit einer gleichzeitig verminderten p63 Expression, ein Regulator in asymmetrischer Zellteilung. Unsere Ergebnisse zeigten eine Verbindung des epidermalen IGF-1R/IR Signalweges mit dem Zellzyklus und asymmetrischer Zellteilung, durch welche es die Anzahl der suprabasalen Zellschichten in der IFE regulieren kann. IGF-1R/IR sind folglich wichtige Regulatoren der epidermalen Morphogenese, des Zellteilungspotentials, der IFE Vorläuferzellen und bei asymmetrischer Zellteilung.

Contents

1 Introduction

1.1 Mammalian skin

This study has dealt with questions regarding how skin morphogenesis, homeostasis and pathogenesis are regulated. Therefore, I would like to introduce the structure of the mammalian skin.

The mammalian skin is the largest organ of the body and contains many specialized cells and structures. It is composed of two distinct compartments, the dermis and the epidermis, separated by the dermal-epidermal junction called the basement membrane. The primary function of the basement membrane is to anchor down the epithelium to its loose connective tissue underneath. This is achieved by cell-matrix adhesions through cell adhesion molecules like integrins. The outermost layer of the skin, the epidermis, is a stratifying epithelium that serves as a protective barrier against environmental factors like UV, pathogens and prevents water loss. It is a complex self renewing organ that consists of the interfollicular epidermis (IFE) and epidermal appendages, such as hair follicles and sebaceous glands (Jones and Simon et al. 2008). As shown in Figure 1, the IFE is organized into four distinct layers of keratinocytes which are characterized by different differentiation markers. The basal layer of the IFE (stratum basale) is composed of proliferating, undifferentiated keratinocytes that are characterized by expression of the intermediate filament pair Keratin14 (K14) and Keratin5 (K5) (Watt 2001). In response to mostly undefined stimuli keratinocytes stop dividing and exit the basal layer to the spinous layer while changing from K14/K5 expression to Keratin1 (K1) and Keratin10 (K10) intermediate filament pair expression. The cells migrate upwards through the spinous layer (stratum spinosum), granular layer (stratum granulosum) which is characterized by loricrin expression and finally the stratum corneum where cells lose their nucleus and become corneocytes (Fuchs 2007). Once the keratinocytes reach the stratum corneum they are gradually shed and are replaced by younger cells pushed up from below (Fig.1).

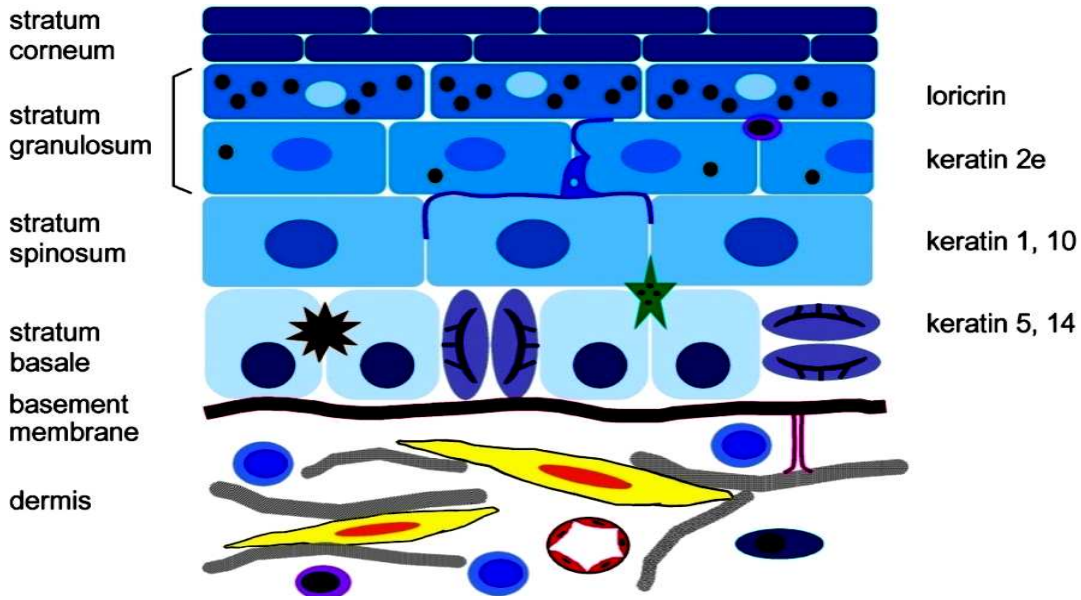

Figure 1: Simplified scheme of the skin.

The skin is composed of two distinct compartments, the dermis and the epidermis, separated by the dermal-epidermal junction that includes a basement membrane. The interfollicular epidermis (IFE) is organized into four distinct layers of keratinocytes; the stratum basale consists of proliferating keratinocytes and express intermediate filament pair K14/K5. The stratum spinosum this is characterized by the expression of the intermediate filament pair K10/K1. The stratum granulosum, expressing loricrin and K2e and finally the cells in the stratum corneum lose their nucleus and become corneocytes

1.2 Progenitor cells in the interfollicular epidermis (IFE)

Continuous renewal of the interfollicular epidermis (IFE) is crucial for organisms to maintain and restore the skin barrier that protects them from external challenges and dehydration. This lifelong process of self-renewal is driven by a high proliferative capacity of progenitor cells which under physiological conditions reside in the basal layer of the interfollicular epidermis coordinating the balance between proliferation and differentiation. (Clayton et al. 2007; Ito et al. 2005; Kaur, 2006; Levy et al. 2005; Watt et al. 2006).

Hair follicle stem cells reside in the bulge of the hair follicle and have well characterized molecular markers (Morris et al. 2004; Tumbar et al. 2004). In contrast, the spatial localization and molecular identity of murine IFE progenitor cells are less clearly defined (Jones and Simons, 2008; Kaur, 2006). In human skin interfollicular progenitor cells in the basal layer are distinguished by differential expression of

markers such as β1 integrin, the Notch ligand Delta and Desmoglein3 but none of these appear to spatially identify a progenitor cell population in the mouse IFE (Jones and Simons, 2008). Similarly, the identity of the niche of IFE progenitor cells is unknown, although it is likely that dermal fibroblasts constitute part of the niche, as has been shown for the hair follicle stem cells located in the bulge (reviewed in (Jones and Wagers, 2008). In the epidermis several downstream mediators, such as p63, the small GTPase Rac and c-Myc, have been implicated in the determination and/or maintainance of interfollicular epidermal progenitor cells and their proliferative potential (Arnold and Watt, 2001; Benitah et al. 2005; Castilho et al. 2007; Koster et al. 2004; Senoo et al. 2007; Waikel et al. 2001). However the extracellular signals and their receptors that regulate these mediators and processes in progenitor cells have remained largely elusive. In humans and mice differential expression of α6 and β1-integrin, receptors for extracellular matrix, are hallmarks associated with differential proliferative potential of basal interfollicular cells (reviewed in Kaur, 2006). This suggests that cell-extracellular matrix adhesion may be one mechanism by which the environment can regulate progenitor cell maintenance.

1.3 Epidermal stratification during embryogenesis

In mice, epidermal stratification is executed over approximately 10 days, from E8.5 to E18.5. It proceeds in multiple stages from a single-layered epithelium on the surface of the ectoderm which expresses keratin 18 (Moll, Franke et al. 1982). At embryonic day E9.5 stratification starts with the formation of the periderm around the upper limb buds and the expression of the stratification markers K5 and K14. Subsequently, at embryonic day E15.5 the epidermis becomes thicker and forms the spinous and granular layer with the concomitant expression of the differentiation markers K10, K1 and loricrin (Bickenbach, Greer et al. 1995). At E16.5 the suprabasal layers further increase and at E18.5 epidermal stratification and maturation is complete and therefore no longer requires the presence of the periderm (Koster and Roop 2007) (Fig.2).

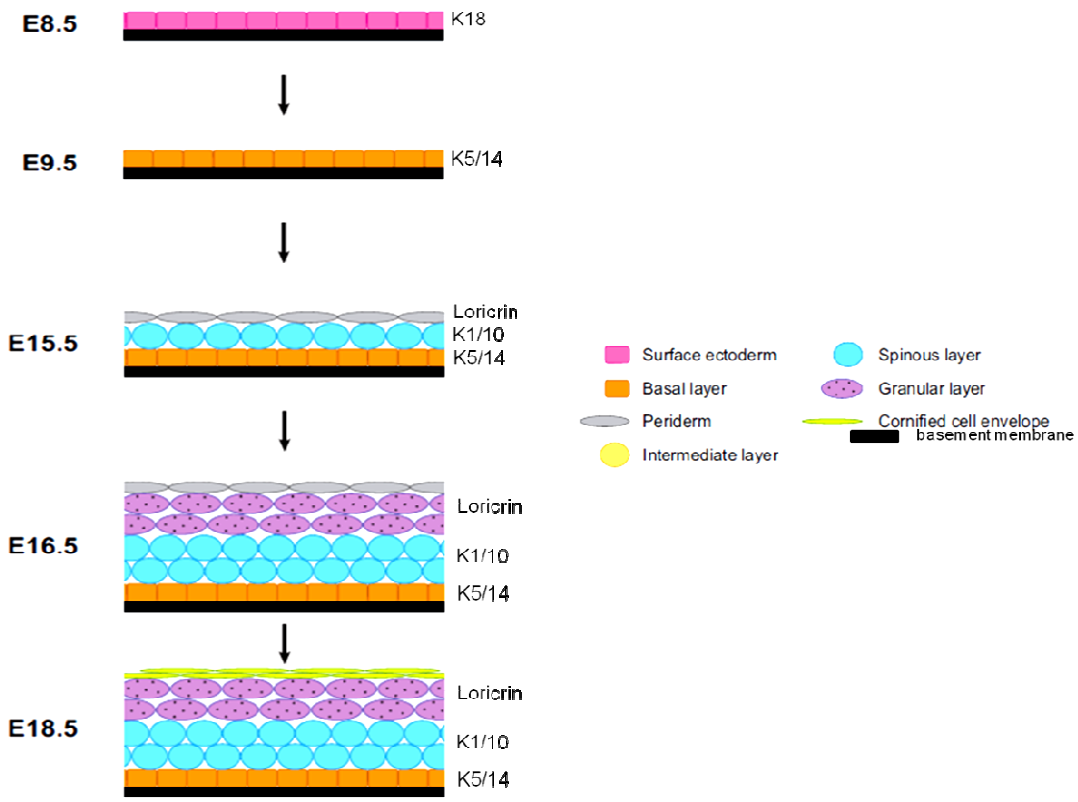

Figure 2: Epidermal stratification during embryonic development

In mice, epidermal stratification takes place from E8.5 to E18.5. The different compartments required for the formation of each layer are highlighted on the right side. The first transition required for epidermal development is epidermal specification, which occurs at approximately E8.5 and results in the induction of K18 in the surface ectoderm. At E9.5 K18 expression switched to K5/K14 intermediate filament protein, specifically expressed in embryonic and mature basal keratinocytes. At E15.5 spinous and granular layer are formed, expressing K1/K10 and loricrin proteins. At E16.5 spinous and granular layers further increased and finally at E18.5 the stratum corneum (green line) is formed and stratification is complete. Figure is taken from (Koster and Roop 2007)

The switch between proliferating basal cells into differentiated suprabasal cells is not yet well understood. Although recent discoveries have highlighted a number of genes required for terminal differentiation of keratinocytes (Takeda, Takeuchi et al. 1999; Rangarajan, Talora et al. 2001; Nicolas, Wolfer et al. 2003), the question remains how the single-layered surface ectoderm commits to initiate stratification during embryogenesis. *In vitro* studies have led to the view that stratification occurs through the delamination and subsequent movement of epidermal cells (Watt and Green 1982; Watt 1984; Vaezi, Bauer et al. 2002) but cultured keratinocytes lack the polarity

and cubical morphology of basal keratinocytes *in situ*. Previously it has been suggested that two different mechanisms might be involved in epidermal stratification during embryonic development.

1.3.1 p63 as a marker for proliferative potential

The p63 transcription factor, a homologue of the tumor suppressor gene p53, is characterized by six different isoforms under the control of two distinct promoters and three different alternative splicing leading to the production of ΔN- or TA-p63, and alpha, beta or gamma isoforms respectively (Yang, Kaghad et al. 1998). In the epidermis ΔNp63 is much more strongly represented than TAp63 and it remains debatable whether TAp63 is expressed at all. TAp63 is, however, strongly expressed in oocytes (Suh, Yang et al. 2006; Livera, Petre-Lazar et al. 2008).

ΔNp63 isoform expression during epidermal development has been detected by RT-PCR and is expressed in the surface ectoderm prior to stratification at embryonic day E8.5 and continues to be expressed during embryonic development (Kai-Hong, Jun et al. 2007). As the epidermis matures p63 expression becomes restricted to the basal layer and in adult tissues p63 is expressed in stratified epithelia, whereas its expression is absent from single-layered epithelia (Yang, Kaghad et al. 1998). Mice lacking all p63 isoforms have disturbed epidermal development (Mills, Zheng et al. 1999) or squamous epithelia (prostate, urothelium) and fail to form a stratified epidermis resulting in lack of barrier formation, consequent dehydration, and death within hours after birth (Mills, Zheng et al. 1999; Yang, Schweitzer et al. 1999). Furthermore, p63 has been implicated in proliferative potential by maintaining stem cells in stratified epithelia. It has been demonstrated that p63 is strongly expressed in epithelial cells with high clonogenic and proliferative capacity and stem cells lacking p63 undergo a premature proliferative rundown. Additionally, the authors showed p63 is dispensable for both the commitment and differentiation of the stem cells during tissue morphogenesis (Senoo, Pinto et al. 2007).

In addition p63 induced several genes involved in cell cycle progression, thus positively controlling proliferation. Down regulation of p63 causes cell cycle arrest in keratinocytes that is p53 dependent (Lee and Kimelman 2002; Truong, Kretz et al. 2006). In contrast, p63 also directly suppressed a subset of genes encoding for late

differentiation markers, while affecting other genes indirectly (Antonini, Dentice et al. 2008; Della Gatta, Bansal et al. 2008).

1.3.2 Asymmetric cell division during embryonic development

Another mechanism that has been implicated in the regulation of epidermal stratification is asymmetric cell division. An asymmetric cell division produces two daughter cells with different cellular fates. This is in contrast to symmetric cell division, which gives rise to daughter cells of equivalent fates. This process is important to generate cell diversity during the development of multicellular organisms, as well as for stem/progenitor cell self-renewal during embryonic development and in adults. Asymmetric cell division has been widely studied in organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Betschinger and Knoblich 2004; Cowan and Hyman 2004; Roegiers and Jan 2004). In flies and worms asymmetric cell division can be subdivided into three steps (Fig.3). First, the mother cell becomes polarized through polarity proteins like Par3/Par6/aPKC complex and LGN proteins, which is usually involved in reorganization of the actin/myosin network and therefore is important for spindle positioning (a). Second, fate determinants like Numb are segregated towards the apical-basal region of the polarized mother cell (b) and third, the mitotic spindle is aligned such that cleavage correctly partitions determinants into two daughter cells (c) (Gonczy 2008). When the anaphase spindles are positioned asymmetrically in the mother cell, daughter cells differ in fate and sometimes in size (d), as shown in one-cell *Caenorhabditis elegans* embryos and in *Drosophila melanogaster* neuroblasts (Betschinger and Knoblich 2004; Cowan and Hyman 2004; Roegiers and Jan 2004).

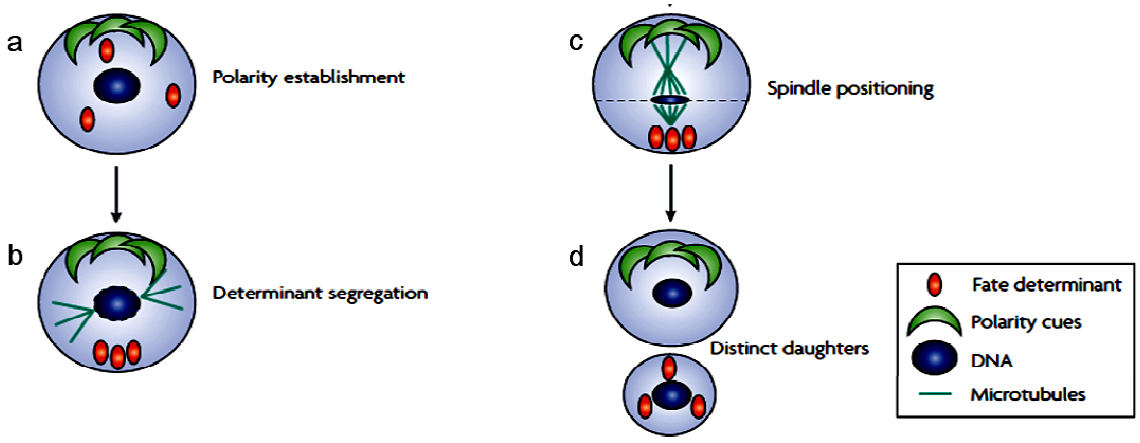

Figure 3: Asymmetric cell division in flies and worms.

Four principal steps ensure asymmetric cell division. (a) The mother cell exhibit cell fate determinants and polarity proteins. (b) In prometaphase spindle formation is activated and fate determinants and polarity proteins are segregated towards the apical-basal region of the cell (dotted line around the nucleus indicates nuclear envelope breakdown). (c) The mitotic spindle is aligned and is ready for dividing into two daughter cells at the equatorial line (dotted line). Cell fate determinants and polarity proteins are lying at opposite poles. (d) Distinct daughter cells differ in cell fate and cell size. Figure is taken from (Gonczy 2008)

In contrast, the function of asymmetric and symmetric cell division in mammals is not well defined. Studies of asymmetric cell division during all phases of cortical neurogenesis in mammalian neuroepithelia reveal a relationship between spindle orientation, cell fate and polarity, but the mechanism is not entirely clear (Knoblich 2001) (Konno, Shioi et al. 2008). Another study revealed a function of asymmetric cell division in epidermal stratification and differentiation (Lechler and Fuchs 2005). Lechler et al. showed that in predominantly single-layered areas of E12.5 epidermis, 92% of divisions occurred symmetric to the basement membrane but from E15.5 onwards through postnatal development when the epidermis becomes thicker due to the formation of spinous and granular layers, more than 70% of spindles were oriented asymmetrically to the basement membrane. By examining p63-null embryos, the authors also could show a functional link between the transcription factor p63 and asymmetric cell division (Lechler and Fuchs 2005). The epidermis of these embryos showed disturbed stratification and almost complete loss of asymmetric cell division in the epidermal basal layer when examined at E16.5 (Lechler and Fuchs 2005). Moreover, Lechler and Fuchs could show that polarity proteins like Par3 and LGN are involved in asymmetrical arrangement in mammalian epidermis as well. They revealed a relationship between asymmetric cell divisions, stratification and an apical organization of LGN and Par3 in the epidermis. Finally they could establish a role for adhesion molecules and the basement membrane in LGN complex distribution and in proper spindle orientation.

Overall, these results suggest that asymmetric cell division may regulate epidermal stratification, perhaps through a p63 dependent activity.

1.4 The cell cycle

A crucial step of asymmetric cell division is the orientation of the spindle with the axis of polarization during the mitotic phase of the cell cycle. During each division, cells complete an ordered series of events that collectively are called the cell cycle. The basic components of this machinery are conserved in all eukaryotes.

1.4.1 Cell cycle stages

The cell cycle consists of four distinct phases and the activation of each phase is dependent on the proper progression and completion of the previous one. The G1 phase, also called the growth phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication (Smith and Martin 1973). Duration of G1 is highly variable, even among different cells of the same species. The S phase starts upon the onset of DNA synthesis. During this phase the DNA is duplicated, though the ploidy of the cell remains the same. Next, cells enter the G2 phase where significant biosynthesis occurs, mainly involving the production of microtubules, which are required during the process of mitosis. Mitosis is the process by which a eukaryotic cell separates the duplicated chromosomes in the cell into two identical sets to the different poles that will represent the two new daughter cells (De Souza and Osmani 2007). It is generally followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal shares of these cellular components. Mitosis and cytokinesis together define the mitotic phase of the cell cycle, which accounts approximately 10% of total cell number of the cell cycle. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G0 phase (Fig.4).

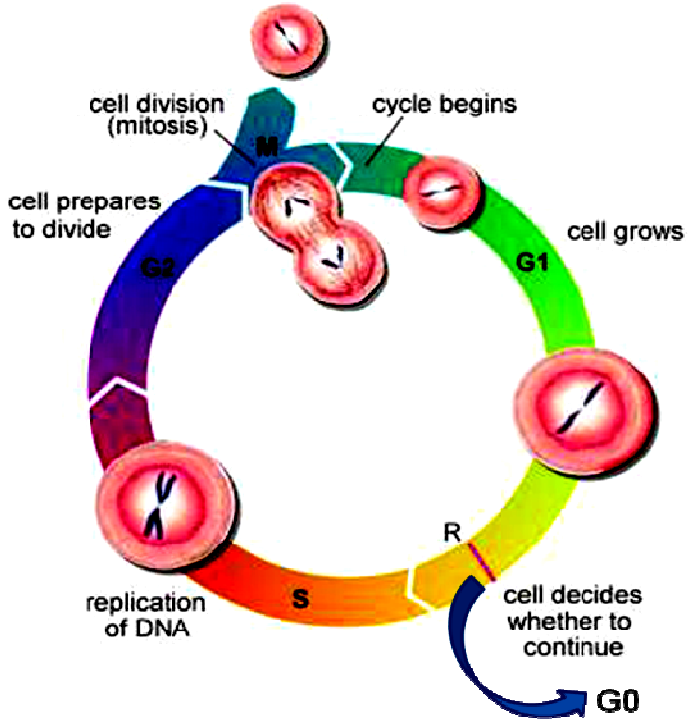

Figure 4: Cell cycle diagram.

G1 phase: Metabolic changes prepare the cell for division. At a certain point - the restriction (R) point - the cell is committed to division and moves into the S phase or stop division and moves into G0. *S phase*: DNA synthesis replicates the genetic material. Each chromosome now consists of two sister chromatids. *G2 phase*: Metabolic changes assemble the cytoplasmic materials necessary for mitosis and cytokinesis. *M phase*: A nuclear division (mitosis) followed by a cell division (cytokinesis). Taken from http://kirschner.med.harvard.edu/files/html/research.shtml

1.4.2 Mitosis

The process of mitosis is complex and highly regulated. The sequence of events is divided into phases, corresponding to the completion of one set of activities and the start of the next. These stages are prophase, prometaphase, metaphase, anaphase and telophase (Nigg, Blangy et al. 1996) .

At the onset of prophase chromatin condenses into a highly ordered structure, the chromosome. Since the genetic material has already been duplicated earlier in S phase, the replicated chromosomes have two sister chromatids, bound together by the cohesion complex. Centrosomes are structures that coordinate the cytoplasmic microtubule network and serve as the major microtubule-organizing centers (MTOC) in mammalian cells. The MTOC harbors a number of protein complexes, including the γ–tubulin ring complex which acts as a template for microtubule nucleation. The

main function of MTOC is to organize the mitotic spindle apparatus, separating the chromosomes during cell division. In the next two phases the nuclear envelope breaks down to allow the microtubules to reach the kinetochores on the chromosomes. The elongated microtubules can attach the kinetochores, leading to the accumulation of the chromosomes along the metaphase plate or equatorial plane (Winey, Mamay et al. 1995). When these events correctly take place the cell proceeds to anaphase.

In this phase, sister chromatids are separated and microtubules and chromosomes are pulled apart to opposite ends of the cell. At the end of anaphase, the cell has succeeded in separating identical copies of the genetic material to two opposite poles. Finally in telophase and cytokinesis corresponding sister chromosomes attach at opposite ends of the cell and a new nuclear envelope is formed which leads to the cell division of two daughter cells (Fig.5)

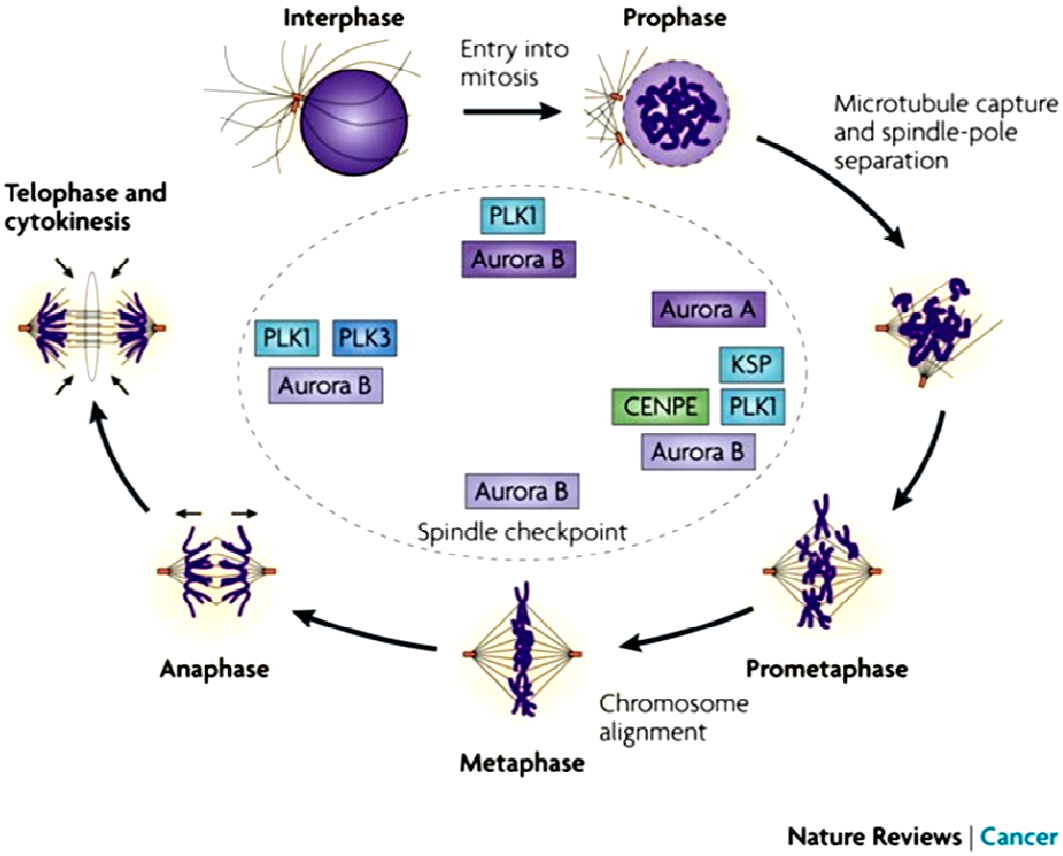

Figure 5: The phases of mitosis.

The progression of mitosis through the canonical morphological stages is shown, with specific protein functions highlighted. Kinesin spindle protein (KSP) is required to establish mitotic spindle bipolarity by driving centrosome separation. Centromeric protein E (CENPE) is required for accurate chromosome congression at metaphase during mitosis. Aurora A is crucial for centrosome maturation and separation during early prophase. Aurora B is a member of the chromosomal passenger complex (CPC) and is involved in histone H3 phosphorylation, chromosomal condensation, chromosomal alignment on the metaphase plate, bi-polar centromere-microtubule attachments, spindle checkpoint and cytokinesis. During mitosis, Polo-like kinase 1 (PLK1) is involved in centrosome maturation and formation of the mitotic spindle. PLK1 is also required for exit from mitosis and the separation of sister chromatids during anaphase. Taken from Jackson et al. Nature Reviews Cancer 7, 107–117 (February 2007) | doi: 10.1038/nrc2049

1.4.3 Network signaling during mitosis

The dynamic assembly of the mitotic spindle is well characterized and has been reviewed many times (Nigg, Blangy et al. 1996; Wittmann, Hyman et al. 2001; Karsenti and Nedelec 2004). Most compounds that perturb the function of the mitotic spindle have turned out to bind microtubules but there are many additional proteins that have crucial roles in the mechanics of mitosis and in progression through the mitotic cell-cycle checkpoint. Some of these proteins are highlighted in Figure 5 such as AuroraA, B and Polo like (PLK) kinases. There are three Aurora kinase family members in mammalian cells AuroraA, B and C, with AuroraA being more evolutionarily distant from the very closely linked B and C members. AuroraA and B are ubiquitously expressed in all dividing cells, whereas Aurora C expression seems to be restricted to the testes (Keen and Taylor 2004).

AuroraA accumulates and peaks in abundance and kinase activity at the G2/M phase of the cell cycle, where it has a crucial role in centrosome maturation and separation during early prophase. The AuroraA mRNA and protein levels are found to be increased in many human epithelial tumour types including breast, bladder, colon, gastric, hepatocellular, ovarian and pancreatic carcinomas, with increasing severity of disease frequently associated with increased expression (Keen and Taylor 2004). Therefore substrates of AuroraA are of significant interest including e.g. the tumor suppressor p53. Studies using siRNA and selective small-molecule inhibitors of

AuroraA lead to cells with severely defective spindle morphology (Hannak, Kirkham et al. 2001; Giet, McLean et al. 2002; Wysong, Chakravarty et al. 2009).

AuroraB also accumulates during mitosis, with peak kinase activity occurring slightly later than AuroraA. AuroraB as a member of the chromosomal passenger complex (see above) is involved in chromosomal condensation, chromosomal alignment on the metaphase plate, bipolar centromere microtubule attachments, spindle checkpoint and cytokinesis (Adams, Carmena et al. 2001; Jackson, Patrick et al. 2007). siRNA and small molecule inhibitors of AuroraB abrogate the mitotic spindle checkpoint and cause premature mitotic exit without completion of cytokinesis, eventually leading to apoptosis or occasionally senescence (Gizatullin, Yao et al. 2006) (Adams, Maiato et al. 2001; Ditchfield, Johnson et al. 2003; Hauf, Cole et al. 2003).

The polo-like kinases (PLKs) are evolutionarily conserved serine or threonine kinases that have crucial roles in regulating cell-cycle processes in diverse organisms from yeast to mammalian cells (Barr, Sillje et al. 2004). In addition, PLK1 is the human homologue of *Drosophila* polo, which is involved in mitosis progression. During mitosis PLK1 has been shown to have roles in centrosome maturation and microtubule dynamics involved in the formation of the mitotic spindle. Furthermore, it is also involved in the exit of cells from mitosis by phosphorylating and activating subunits of the anaphase-promoting complex. PLK1 also phosphorylates cohesin proteins that hold sister chromatids together (as discussed above), exposing separase cleavage sites and enabling the separation of sister chromatids during anaphase (Sumara, Gimenez-Abian et al. 2004; Neef, Gruneberg et al. 2007; Haren, Stearns et al. 2009).

1.4.4 Cell cycle checkpoints

Cell cycle checkpoints are control mechanisms that ensure the fidelity of cell division in eukaryotic cells. Such flawless operation of the cell cycle is important not only for the maintenance of viability but also to prevent mutations and genetic instability. Three different checkpoints exist in the cell cycle, the G1, G2 and the mitotic checkpoint (also known as spindle checkpoint). These checkpoints verify whether the processes at these phases of the cell cycle have been accurately executed before progression into the next phase (Pietenpol and Stewart 2002).

The first checkpoint is located at the end of the G1 phase, just before entry into S phase, making the key decision of whether the cell should divide, delay division, or enter a resting stage. Under normal condition many cells stop at this stage and enter a resting state called G0 (Fig.4).

The second checkpoint is located at the end of G2 phase before the start of mitosis. In order for this checkpoint to be passed, the cell has to check a number of factors to ensure cells are ready for mitosis. If this checkpoint is passed, the cell initiates the many molecular processes that signal the beginning of mitosis.

An important function of the G2/M checkpoint is to assess DNA damage, which is detected by sensor mechanisms. When DNA damage is found, the checkpoint uses a signal mechanism to either stop the cell cycle until repairs are made or, if repairs can't be made, to target the cell for destruction via apoptosis or senescence. One important regulator of this process is p53, a tumor suppressor protein that is able to block the cell cycle after DNA damage and cause apoptosis when damage is irreversible (Pellegata, Antoniono et al. 1996). Due to this crucial function p53 was suitably dubbed the “guardian of the genome” (Levine and Oren 2009).

The last checkpoint, the so-called spindle checkpoint, occurs in metaphase when all the chromosomes should have aligned at the mitotic plate and be under bipolar tension. The tension created by this bipolar attachment initiates the anaphase entry by activating the anaphase promoting complex.

The anaphase-promoting complex (APC), also called cyclosome, is a complex of several proteins activated during mitosis to initiate anaphase. The APC is an E3 ubiquitin ligase that marks target proteins for degradation by the 26S proteasome.

The spindle checkpoint inhibits the APC until all sister-kinetochores are correctly attached to opposite poles of the mitotic spindle. When all kinetochores are properly attached, the spindle checkpoint is silenced and the APC becomes active. Activated APC then targets securin for degradation. Securin inhibits a protease called separase, which cleaves cohesins allowing anaphase onset (Peters 2002; Uhlmann 2003; Blow and Tanaka 2005). In addition, the so called chromosomal passenger complex, consisting of AuroraB kinase, Inner Centromere Protein (INCENP), borealin and survivin, plays a crucial role to promote spindle checkpoint activity during metaphase. Within the chromosomal passenger complex AuroraB is the enzymatic core that is activated and guided to its specific locations in the mitotic cell by

INCENP, borealin and survivin (Klein, Nigg et al. 2006; Vader, Medema et al. 2006). It has been shown that especially AuroraB regulates chromosome alignment and triggering mitotic arrest through the spindle checkpoint by correcting faulty chromosome-spindle interactions (Vader, Maia et al. 2008). Cells will then continue with the cell cycle after correct chromosome alignment.

1.5 Signaling through IGF-1 and insulin receptor

The insulin-like growth factor 1 (IGF-1R) and insulin receptors (IR) are transmembrane receptor tyrosine kinases, which are widely expressed in many tissues and cell types. The insulin-like growth factor (IGF) axis is involved in the coordinated function of IGF-1, IGF-2 and insulin ligands and exhibit three cell surface receptors, the IR, IGF-1R and IGF-2R (Baserga, Hongo et al. 1997) (Pollak, Schernhammer et al. 2004). Insulin and IGF peptides have different affinities for Insulin Receptor (IR), IGF-1R and IGF-2R, e.g. IGF-1 binds with high affinity to the IGF-1R and with a 10-fold lower affinity to the IR but do not bind to the IGF-2R. IGF-2R is a transmembrane protein, but as yet has no reported tyrosine kinase receptor acivity (Fig.6).

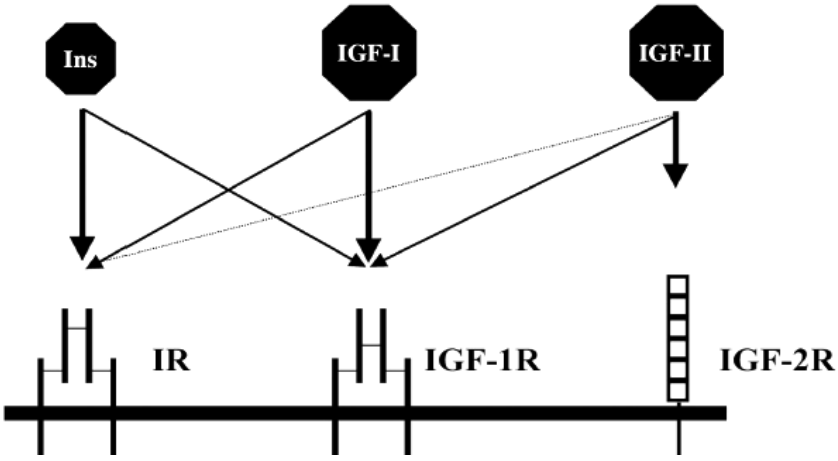

Figure 6: Binding affinity of IGF-1, IGF-2 and Insulin to their related receptors.
Insulin and IGF peptides have different affinities for Insulin Receptor (IR), IGF-1R and IGF-2R. Only IR and IGF-1R have transmembrane receptor tyrosine kinase activity. Thicknesses of the arrows represent the affinity of a ligand for that receptor. Figure taken from Julien-Grille et al. 2000 "The Role of Insulin-like Growth Factors in the Epithelial to Mesenchymal Transition"

The IGF-1R signaling plays an important role in normal growth and development (Liu, Baker et al. 1993) and is implicated in mediating many aspects of the malignant phenotype in a variety of human malignancies (Khandwala, McCutcheon et al. 2000; Moschos and Mantzoros 2002) (Bohula, Playford et al. 2003). The IGF-1R gene is located on chromosome 15q26 and encodes a single polypeptide of 1367 amino acids that is constitutively expressed in most cells. The IR gene is located on chromosome 19p13 and encodes 1382 amino acids. The IGF-1R and insulin receptor (IR) share approximately 70% amino acid identity, and their genes exhibit striking homology in terms of size and exon structure (Ullrich, Gray et al. 1986). Both receptors are synthesized as single-chain pre-proreceptors, with a 30-aa-residue signal peptide that is co-translationally cleaved. The proreceptors cursor is then glycosylated, proteolytically cleaved, and cross linked by cysteine bonds to form a functional transmembrane αβ chain.

The mature IR and IGF-1R are large transmembrane glycoproteins (~300-350kDa) composed of two α (130–135 kDa) and two β (90–97 kDa) subunits. The α- and β-subunits are proteolytically cleaved from the single common proreceptor and covalently linked into dimers by disulphide bonds. The putative ligand binding pockets are located in the cysteine rich portion of the extracellular α subunits whereas the β subunit contains tyrosine kinase domain, ATP and substrate binding pockets and autophosphorylation sites (Riedemann and Macaulay 2006). Binding of ligand to the IR or IGF-1R leads to activation of the kinase and subsequent autophosphorylation on different tyrosines in the kinase domain (Fig.7), followed by phosphorylation of juxtamembrane and carboxyterminal tyrosines. This leads to the recruitment of specific docking intermediates, including insulin-receptor substrate-1 (IRS-1), Shc and 14-3-3 proteins on specific phosphorylated residues (Baserga, Hongo et al. 1997) (Pollak, Schernhammer et al. 2004). Thereby linking the IR/IGF-1R to diverse signaling pathways those regulate growth, proliferation, differentiation and apoptosis. For example, following phosphorylation of IRS-1 by the activated IGF-1R, PI3K is activated by binding of regulatory subunit to IRS-1 (Baserga et al. 1997). This interaction leads to an increase in the levels of phosphatidylinositol 3, 4, 5-triphosphate (PIP3), which leads to recruitment and activation of phosphoinositide dependent kinase-1 (PDK-1) and AKT protein kinase B. The activated AKT induces

inhibitory phosphorylation of pro-apoptotic factors like members of the fork head transcription factor family e.g. Foxo1 (Brazil, Yang et al. 2004) (Fig.7).

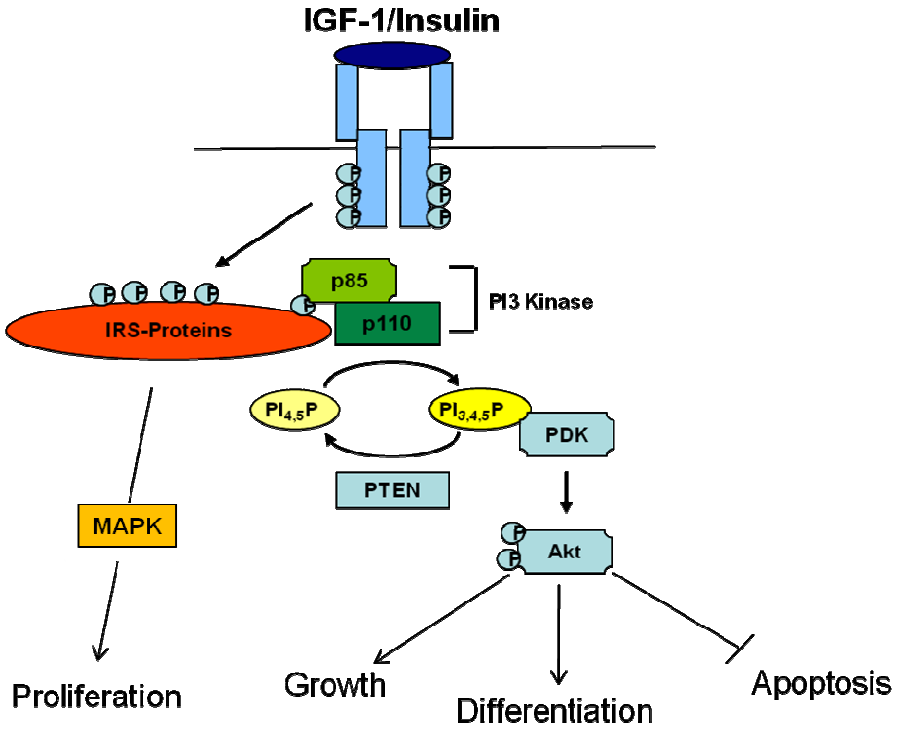

Figure 7: Simplified scheme of IGF-1R/IR signaling pathway.
Insulin/IGF-1 ligand binding to the IR/IGF-1R leads to autophosphorylation on different tyrosines in the kinase domain, followed by recruitment of IRS-1. A well known downstream target of IGF-1R/IR is PI3K which is activated by binding its regulatory subunit to IRS-1. This interaction leads to an increase in the levels of phosphatidylinositol 3, 4, 5-triphosphate (PIP3), which leads to recruitment and activation of phosphoinositide dependent kinase-1 (PDK1) and AKT/protein kinase B. Another pathway activating through IRS-1 signaling is the MAPK pathway. These molecules link the IGF1R to diverse signaling pathways, allowing induction of growth, proliferation, differentiation and apoptosis.

The importance of the IGF-1R in normal mammalian development is clear from studies in mice lacking functional receptors. IGF-1R null mice are 45% of the size of wild type littermates at birth and die shortly due to severe organ hypoplasia (Liu, Baker et al. 1993). Mouse embryonic fibroblasts cultured from IGF-1R null mice grow slower than wild-type fibroblasts and are unable to proliferate under anchorage-independent conditions (Sell, Ptasznik et al. 1993). Furthermore, the IGF-1R is frequently over expressed in tumors, including melanoma and cancer in different

tissues (Hellawell, Turner et al. 2002) and can result in the loss of the tumor suppressor p53 (Bruchim, Attias et al. 2009).

The main function of insulin receptor activation is inducing glucose uptake. For this reason "insulin insensitivity“ or a decrease in insulin receptor signaling leads to diabetes mellitus type 2 - where cells are unable to take up glucose, and the result is hyperglycemia which is an increase in circulating glucose (Clauser 1994).

Mice lacking insulin receptors are born with normal features but develop early postnatal diabetes and die of ketoacidosis (Accili, Drago et al. 1996). Combined knockout studies of insulin and IGF-1 receptors indicate that the insulin receptor also promotes embryonic growth (Kitamura, Kahn et al. 2003).

1.6 Pathogenic role of IGF-1R/IR signaling in the skin

Due to the diverse function and ubiquitous expression of IGF-1R and IR, the pathway has been implicated in a variety of human malignancies in many different tissues. Because my thesis has dealt with how skin morphogenesis and homeostasis is regulated, we have focused our attention only on the role of IGF-1R/IR signaling in the skin.

The health and proper functioning of the skin is highly dependent on the synergistic interaction between the dermis and epidermis. Factors regulating these interactions are IGF-1 and insulin (Barreca, De Luca et al. 1992; Tavakkol, Elder et al. 1992). In human skin, keratinocytes express the IGF-1R and IR but they do not synthesize IGF-1 or insulin (Barreca, De Luca et al. 1992; Tavakkol, Elder et al. 1992). Therefore, dermal fibroblasts could support the proliferation of keratinocytes in the epidermis by secreting IGF-1 and insulin (Barreca, De Luca et al. 1992; Tavakkol, Elder et al. 1992). The IGF-1R has also been shown to be important in normal epidermal differentiation (Sadagurski, Yakar et al. 2006). Therefore the activation of the IGF-1R/IR can influence all stages of epidermal homeostasis.

Recently, it has been shown that altered expression of IR as well as IGF-1R results in impaired wound healing in type 2 diabetes mellitus patients and during skin cancer development.

1.6.1 Impaired wound healing in patients with type 2 diabetes mellitus

Type 2 diabetes mellitus is characterized by insulin resistance and represents the most frequent endocrine disease, affecting more than 6% of western populations (Mokdad, Ford et al. 2003). Thereby it represents a major socio-economical health burden. Lethality results largely from diabetic complications including micro- and macro vascular diseases (Mokdad, Ford et al. 2003). Moreover diabetes is associated with many different skin diseases and a hallmark of diabetes is an impaired wound healing. It is well established that ulcerations, often resulting in amputation, represent serious complications of diabetes mellitus and are associated with significant mortality (Abbott, Carrington et al. 2002). The lifetime risk for a diabetic patient to develop such a complication ranges around 15% (Faglia, Favales et al. 2001; Reiber, Smith et al. 2002). Despite the importance of diabetes, therapeutic interventions to date are limited. Besides wound healing defects, diabetes is also associated with skin barrier breakdown and an increased susceptibility for both bacterial and fungal infections (Ferringer and Miller 2002). Moreover, syndromes of insulin resistance show an increased association with disseminated granuloma annulare which is a chronic skin disease consisting of a rash with reddish bumps arranged in a circle or ring and necrobiosis lipoidica which is a disorder of collagen degeneration with a granulomatous response, thickening of blood vessel walls, and fat deposition (Huntley 1993; Sibbald, Landolt et al. 1996). Yet, it is still not resolved whether diabetic skin complications result as a secondar response to metabolic alterations such as hyperglycemia or directly from impaired insulin action in skin (Brownlee 2001). Along this line it is important to state that functional insulin signaling molecules are expressed in keratinocytes and severe impairment of insulin signaling in wounds of diabetic mouse models have been described (Goren, Muller et al. 2006).

Furthermore, mice with complete knockout of IGF-1R die shortly after birth from respiratory failure and show an abnormally thin and translucent epidermis (Liu, Baker et al. 1993) which is also observed in AKT1/2 double deficient mice (Peng, Haldar et al. 2003). In contrast complete inactivation of the closest relative of IGF-1R, the insulin receptor (IR), did not reveal any obvious macroscopic skin phenotype in mice even though proliferation and differentiation were altered (Wertheimer et al., 2001).

In addition, one major target of activated AKT kinase is the Foxo family of transcription factors keeps the larger family of forkhead class transcription factors. Recent data illustrate that Foxo proteins are regulated by multiple signaling pathways implicated in skin homeostasis, such as tumor necrosis factor α (TNF-α) and the Notch signaling pathway (Lu, Wu et al. 2004; Lu, Sheu et al. 2005). Thus, the link between insulin/IGF and Notch signaling with Foxo transcription factors provides a molecular mechanism balancing skin morphogenesis and homeostasis.

1.6.2 IGF-1R/IR signaling in skin cancer progression and development

The main environmental risk factor for developing skin cancer is exposure to the ultraviolet radiation in sunlight, primarily UV-B wavelengths (Tyrrell 1994; Wikonkal and Brash 1999). Another risk factor is age, approximately 80% of all skin cancers are found in people over the age of 60.

The importance of IGF-1R signaling in skin carcinogenesis is evident from a variety of studies. Transgenic mice over expressing either IGF-1 or IGF-2 in the basal layer of skin epidermis exhibited epidermal hyperplasia, hyperkeratosis and squamous papillomas (Bol, Kiguchi et al. 1997; DiGiovanni, Bol et al. 2000; Bennett, Crew et al. 2003). IGF-2 has recently been implicated in the regulation of clonogenecity of human embryonic stem cells via activation of the IGF-1R (Bendall et al., 2007). Overexpression of IGF-2 in either mouse colon or epidermis also increased the number of proliferative units without a change in cell size, or, in colon, crypt area (Ward et al. 1984) (Bennett et al. 2003). In addition, both insulin and IGFs negatively regulate the Foxo family of transcription factors (Taniguchi et al. 2006) which have been shown to play a central role in the regulation of stem cells (Arden, 2007). Consistently, conventional inactivation of IGF-1R signaling components such as IGF-1R, IGF-1 and/or IGF-2 (Liu et al. 1993), Insulin substrate 1 (IRS1) (Sadagurski et al. 2007) or both of the downstream kinases AKT1 and AKT2 (Peng et al. 2003) in mice each result in a disturbed stratification process generating hypomorphic epidermis although the mechanisms remain unclear.

The control of longevity has also been demonstrated to be critically controlled by insulin/IGF-1R signaling pathway in invertebrate and mammalian animal models (Lin, Hsin et al. 2001; Holzenberger, Dupont et al. 2003) (Garinis, Uittenboogaard et al.

2009). Persistent DNA-damage leads to decreased IGF-1R expression, resulting in cellular IGF-1 resistance, which was also shown in aging animals.

1.7 Epidermal specific inactivation of IR and/or IGF-1R

To examine the cell autonomous role of insulin and IGF-1 signaling in the epidermal compartment of the skin, we specifically inactivated their receptors, either the insulin receptor ($IR^{-/-}$), the IGF-1 receptor ($IGF\text{-}1R^{-/-}$) or both receptors (double knockout or dko) in the epidermis. This was achieved by crossing mice homozygous for the respective floxed alleles (Brüning et al., 1998) of these receptors with mice expressing the Cre recombinase under the K14 promoter (Hafner et al., 2004) and carrying one floxed allele for either the IR and/or IGF-1R. This results in the deletion of the floxed region of IR or IGF-1R or both at the genomic level and absence of protein expression (Fig.8A) in the epidermal compartment. The first characterization, and initial experiments, of these mice was done by Hady Ibrahim.

Mice with epidermal loss of IR were viable and exhibited no macroscopically detectable defects either in the epidermis (Fig.8B) or in hair follicles, as was expected based on the total IR knockout mice (Wertheimer et al., 2001). In contrast, inactivation of the IGF-1R or a combination of IGF1-R and IR (dko) resulted in a fragile, translucent skin, (Fig.8B) a more severe appearance occurring in the dko. All dko mice died prenatally, whereas around 55% of the $IGF\text{-}1R^{-/-}$ mice survived but showed occasional hair loss.

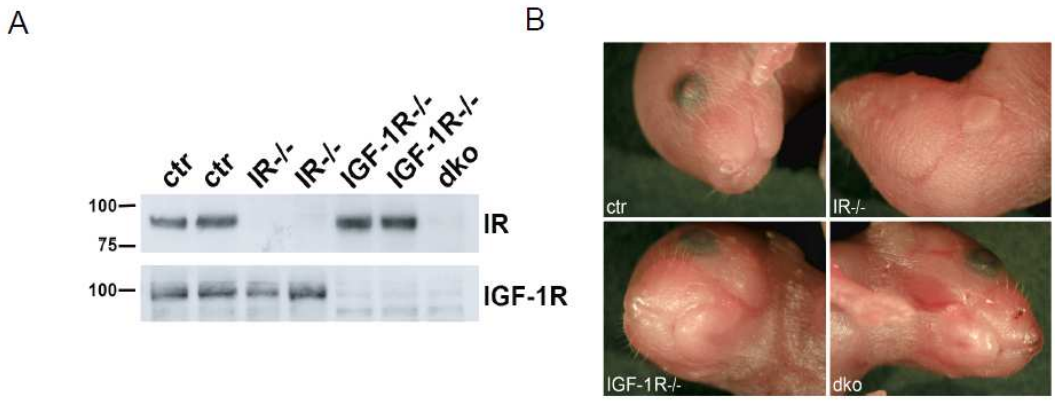

Figure 8: Epidermal inactivation of insulin receptor and IGF-1 receptor affects epidermal morphogenesis.

(A) Western blot analysis on epidermal lysates isolated from ctr, IR-/-, IGF-1R-/- and dko newborn mice using antibodies against either IR receptor or IGF-1R receptor. (B) Macroscopic appearance of control (ctr) mice and mice with an epidermal deletion of IR, IGF-1R or both (dko).

Histochemical analysis revealed a strikingly hypoplastic epidermis in the IGF-1R$^{-/-}$ mice (Fig.8A&B), showing that the previously observed hypomorphic epidermis of the total IGF-1R knockout mice (Liu et al., 1993) results directly from a signaling defect within the epidermis. Surprisingly, even though the IR$^{-/-}$ mice displayed no obvious macroscopic phenotype (Fig.8B), the interfollicular epidermis was significantly thinner than that of control mice (Fig.9B). Deletion of both IR and IGF-1R resulted in a further decrease (Fig.9A&B), revealing that cell autonomous cooperation between insulin and IGF-1 signaling regulates epidermal thickness.

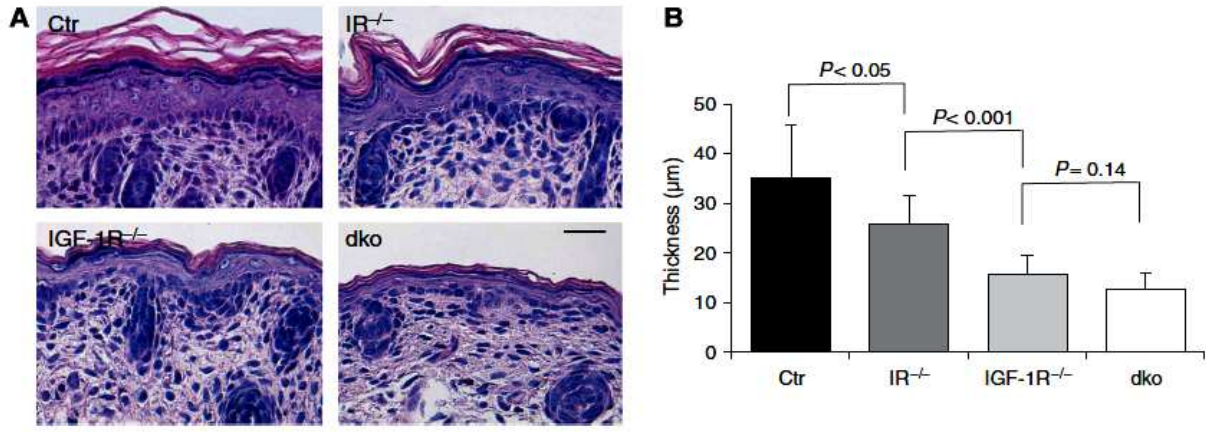

Figure 9: Cooperative and cell-autonomous regulation of epidermal thickness by epidermal insulin and IGF-1 receptor signaling.

(A) H&E staining of paraffin sections from ctr, IR-/-, IGF-1R-/- and dko newborn back skin. Scale bar is 50 µm. (B) Quantification of the thickness of the epidermis (without the stratum corneum) using H&E stained sections of back skin (one representative section is shown in A). N=7 for each genotype.

The hypomorphic, epidermal phenotype was not limited to the epidermis and was observed in other stratifying epithelia that express K14 such as the palate and tooth anlagen. The hypomorphic epidermis did not further detoriate in surviving IGF-1R$^{-/-}$ mice or IR$^{-/-}$ mice (Stachelscheid, Ibrahim et al. 2008). Thus, insulin and IGF-1 receptor signaling cooperatively regulate the number of epidermal cell layers and thereby interfollicular epidermal morphogenesis, with a more significant regulation by IGF-1R compared to IR signaling. Since IR$^{-/-}$ mice showed no obvious hair follicle defects and dko mice died within the first two days, the study focused on the role of IR/IGF-1R in regulating interfollicular morphogenesis.

1.7.1 Normal differentiation in the absence of epidermal IR/IGF-1R

The appearance of a hypo- or hypermorphic epidermis is often associated with impaired differentiation. This was indeed reported in three dimensional skin co-culture systems with keratinocytes and fibroblasts both deficient in IGF-1R (Sadagurski et al., 2006), suggesting that IGF-1R could contribute to the observed reduction in spinous and granular cell layers. To examine if epidermal inactivation of either the IR or the IGF-1R signaling pathway directly impaired differentiation *in vivo* different IFE markers were used. In the IR$^{-/-}$, IGF-1R$^{-/-}$ or dko mice K10 and loricirin expression was observed, with K10 marking the spinous layers and loricrin marking granular layers. However, due to the reduction in spinous and granular layers in knockout mice, the domain was increasingly thinned (Fig.10). The basal layer marker K14 was confined to the basal layer in all mice examined (Fig.10), moreover no obvious difference in integrin α6 staining a marker of the epidermal-basement membrane zone, was observed suggesting normal polarization of basal keratinocytes and contact with the basement membrane (Fig.10).

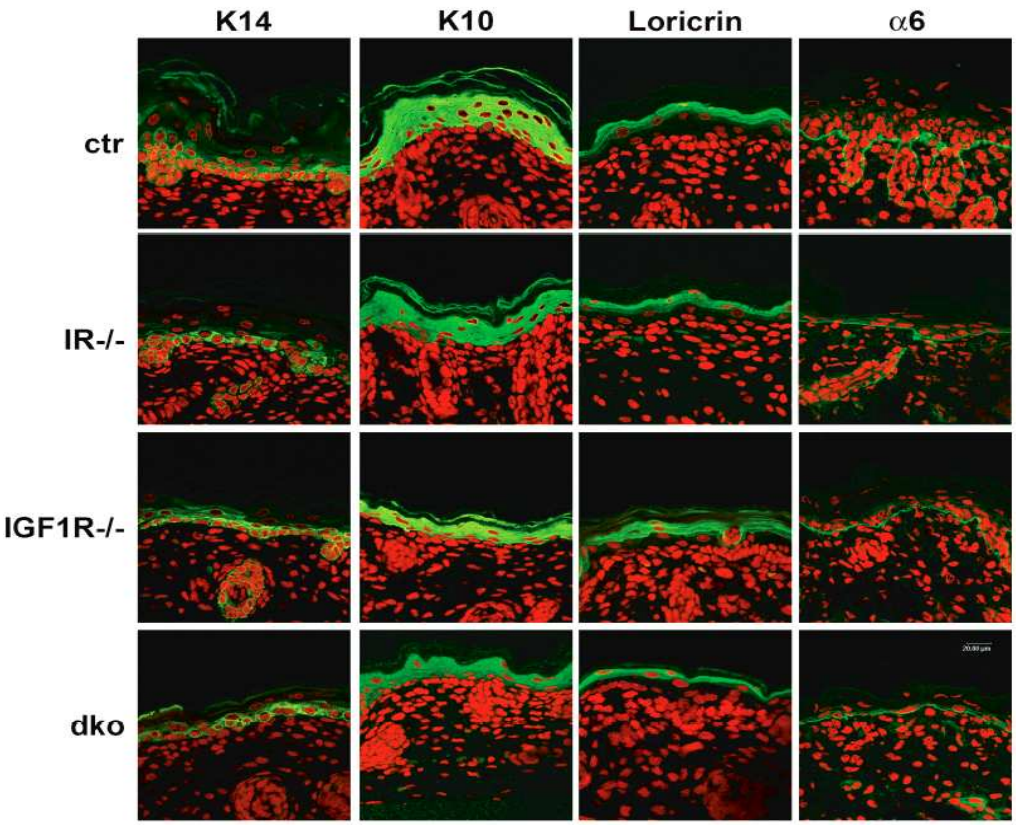

Figure 10: Normal differentiation in newborn mice in the absence of epidermal IR/IGF-1R. Immunofluorescence staining of different differentiation markers K14, K10, loricrin and integrin α6 (green) on paraffin sections isolated from back skin of ctr, $IR^{-/-}$, $IGF\text{-}1R^{-/-}$ and dko newborn mice. Nuclei were counterstained using PI (red). Scale bar is 20µm.

These results show that in $IGF\text{-}1R^{-/-}$ and $IGF\text{-}1R/IR^{-/-}$ epidermis, differentiation markers retain their proper spatial distribution, indicating that the intrinsic differentiation program is not directly affected by loss of epidermal insulin and/or IGF-1 signaling. Fewer suprabasal (spinous & granular) layers were the most distinct defect in the epidermis after disruption of IGF-1R and /or IR.

1.7.2 IR/IGF-1R signaling does not affect apoptosis in newborn epidermis

Both insulin and IGF-1 can promote cell survival by activation of AKT (Pollak et al., 2004; Taniguchi et al., 2006). *In vitro* studies using keratinocytes negative for either IR or IGF-1R revealed changes in AKT signaling and increased apoptosis (Sadagurski et al., 2006; Wertheimer et al., 2001). However, no change in apoptotic activity was seen in the epidermis after inactivation of either IR or IGF-1R alone or

IGF-1R/IR dko as assessed by Tunel assays (Fig.11) or cleaved caspase 3 protein levels (not shown).

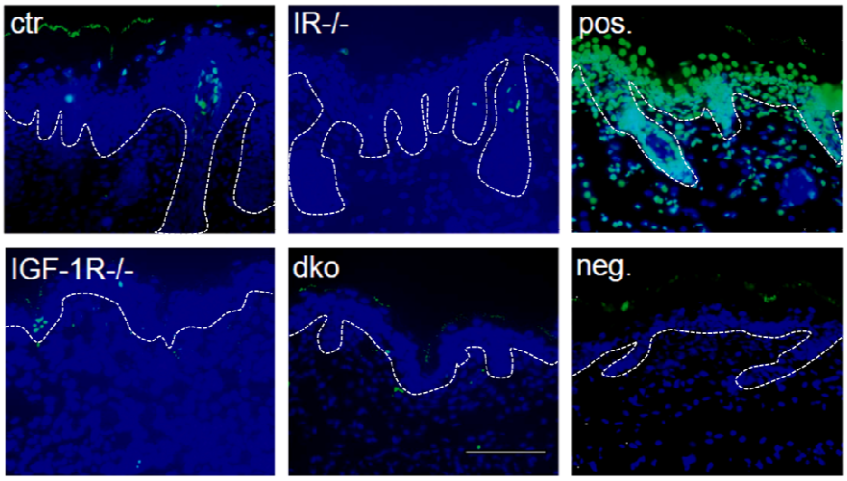

Figure 11: No increased apoptosis in newborn epidermis in the absence of IR/IGF-1R.
Tunel staining (green) on sections isolated from back skin of ctr, IR-/-, IGF-1R-/- and dko newborn mice. Nuclei were counterstained using DAPI (Blue). White line represents the basement membrane, separating epidermis from the dermis. Scale bar is 100 µM.

Although IGF-1R signaling can stimulate AKT kinase activity in keratinocytes (Haase et al., 2003; Sadagurski et al., 2006) relatively little activated AKT was detected under steady-state conditions in isolated control newborn epidermis, and was similar or even slightly increased in the absence of IR, IGF-1R or dko. Localization of phospho-AKT was also unaltered in dko epidermis (not shown). Strikingly, an increase in total levels of AKT was seen upon deletion of IR, IGF-1R or both, suggesting that epidermal keratinocytes attempt to compensate for the loss of IR and/or IGF-1R. As an indirect measure of changes in AKT activity we also assessed the phosphorylation status of its downstream target GSK-3β. Deletion of IR, IGF-1R or both did not obviously alter GSK-3β phosphorylation (Stachelscheid, Ibrahim et al. 2008).

1.7.3 Proliferation is not altered in newborn mice

In the epidermis, over expression of IGF-1 or IGF-2 results in hyperproliferation (Bol, Kiguchi et al. 1997; Bennett, Crew et al. 2003) whereas MAPK activation is altered in keratinocytes deficient for IGF-1R (Sadagurski, Yakar et al. 2006), thus providing a

potential explanation for the hypomorphic epidermis. Surprisingly, no obvious change in the proliferation marker Ki67 could be detected between control, IR-/-, IGF-1R$^{-/-}$ and dko newborn epidermis (Fig.12A). As even stronger decrease in layer number was seen in the stratifying epithelia of the palate and tongue, which may reveal more obviously alterations in proliferation and therefore examinations of Ki67 staining were performed in these tissues. Again, no significant difference in Ki67 positive cells was observed in control versus dko palate and tongue epithelium (Fig.12A&B), perhaps due to compensatory effects from the dermis.

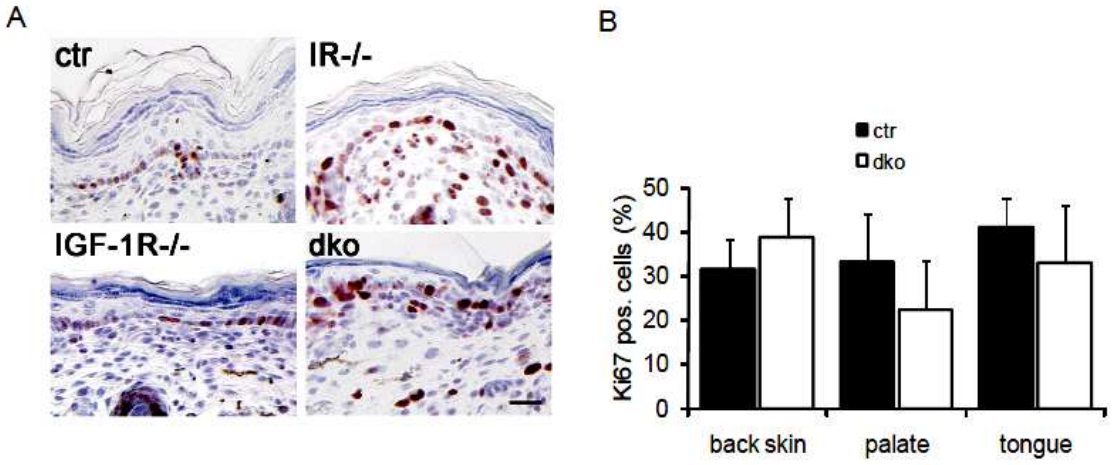

Figure 12: Normal proliferation in newborn mice in the absence of epidermal IR/IGF-1R.
(A) Ki67 staining on sections of back skin isolated from ctr, IR-/-, IGF-1R-/- and dko newborn mice. Scale bar is 100 µM (B). Quantification of Ki67 staining in the basal cells of IFE of control and dko mice in back skin, palate or tongue epithelium. N=5 and P>0.05 for each genotype.

To directly examine the consequences of IR or IGF-1R signal alteration in proliferation, growth assays were performed with primary keratinocytes. Whereas IGF-1R$^{-/-}$ keratinocytes did not grow in the absence of fibroblast feeders, no growth impairment was observed for the IR$^{-/-}$ keratinocytes in comparison to control keratinocytes isolated from littermates (Stachelscheid, Ibrahim et al. 2008). Recently, it was demonstrated that inactivation of Mek1/2, upstream kinases of MAPK, in the epidermis almost completely abrogates MAPK activation in newborn mice. This is associated with a hypomorphic epidermis, similar to that observed in dko mice, and changes in proliferation during embryogenesis (Scholl, Dumesic et al. 2007). However, we could not detect any differences in either total or active MAP Kinase levels in the epidermis of ctr, IR, IGF-1R or IR/IGF-1R negative epidermis (Stachelscheid, Ibrahim et al. 2008). This demonstrates that IR and IGF-1R are not

the crucial activators of the MAPK pathway in murine epidermis, consistent with results in human keratinocytes (Haase, Evans et al. 2003). Together these results show that IGF-1R but not IR signaling regulates proliferation *in vitro* but not *in vivo,* at least in newborn mice.

First characterizations of the mice with epidermal specific deletion of IGF-1R and/or IR showed a cell autonomous function of IGF-1R and IR in the epidermis. Newborn mice developed a thinner epidermis compared to controls, derived from fewer suprabasal layers in the interfollicular epidermis. Surprisingly, no alterations in apoptosis, differentiation and proliferation could be detected in $IR^{-/-}$, $IGF\text{-}1R^{-/-}$ and dko newborn mice.

1.8 Aims of this study

The overall goal of this study was to determine the specific cell autonomous function of IGF-1R and IR in the epidermis and to determine the molecular mechanism by which cell autonomous IR/IGF-1R signaling regulates epidermal morphogenesis.
The specific aims/questions addressed in this study were

1. To further characterize the skin phenotype of the epidermal specific IGF-1R$^{-/-}$, IR$^{-/-}$ and dko mice.

2. Does the loss of epidermal IGF-1R and IR affect stem/progenitor cell behavior in the epidermis and in culture?

3. What are the molecular pathways directly downstream of IR/IGF-1R in the epidermis?

4. How does IR/IGF-1R signaling regulate epidermal stratification during embryonic development?

2 Results

2.1 IR/IGF-1R signaling regulates proliferative potential in keratinocytes

The initial analysis of epidermal specific IR/IGF-1R knockout mice, done by Hady Ibrahim in the laboratory, showed that cell autonomous insulin- and IGF-1 receptor signaling in the epidermis cooperatively regulates epidermal morphogenesis. Surprisingly differentiation, survival or proliferation was not affected by the epidermal specific loss of IR/IGF-1R in newborn mice. To further address how cell autonomous insulin/IGF-1 signaling regulates the number of suprabasal layers during epidermal morphogenesis the colony-forming capacity of primary keratinocytes (Izumi, Tobita et al. 2007) isolated from control, $IR^{-/-}$ or $IGF\text{-}1R^{-/-}$ mice was analyzed. Whereas the number of colonies is an indirect indication for proliferation per se and/or cell-matrix adhesion, the size of the colonies represents the number of cell divisions a single cell can undergo, both of which are a measure of proliferative potential. Large colonies are thought to represent progenitor cells showing high proliferative potential (Izumi, Tobita et al. 2007). It was found that IGF-2 regulates proliferative potential of human embryonic stem cells (Bendall, Stewart et al. 2007). Insulin/IGF may thus similary regulate the proliferative potential of epidermal progenitor cells and this may indirectly control the number of suprabasal layers.

A strong reduction in the size of the colonies was observed in $IGF\text{-}1R^{-/-}$ keratinocytes compared to controls, with an almost complete loss of large-sized colonies (Fig.13, experiment done by Hady Ibrahim). A reduction in colony size was also observed in $IR^{-/-}$ keratinocytes compared to control, but with a less dramatic effect compared to $IGF\text{-}1R^{-/-}$ (Fig.13A&B). This is in line with the *in vivo* results showing that loss of the epidermal IR affects the thickness of the epidermis less severely than loss of IGF-1R (see introduction).

When increasing colony size is plotted as a continuum against accumulative percentage of colonies, two different gradients could be identified in control keratinocytes, one with a relative flat slope that represents 90% of all colonies and another where the steepness of the slope dramatically increases over the last 5–10% (Fig.13C). This indicates the presence of two different cell populations, one that represents over 90% of the colonies that have a similar, relatively low proliferative potential, and a second representing around 5–10% of the colonies with a much

higher proliferative potential. This population likely represents epidermal progenitor cells. When comparing the curve for IGF-1R$^{-/-}$ cells the overall angle of the initial slope is less than that of the control, indicating that proliferative potential is reduced in all cells. In fact, for IGF-1R$^{-/-}$ cells the steepness of the curve remained unchanged, indicating that the population with high proliferative potential is almost completely absent in these cells (Fig.13C).

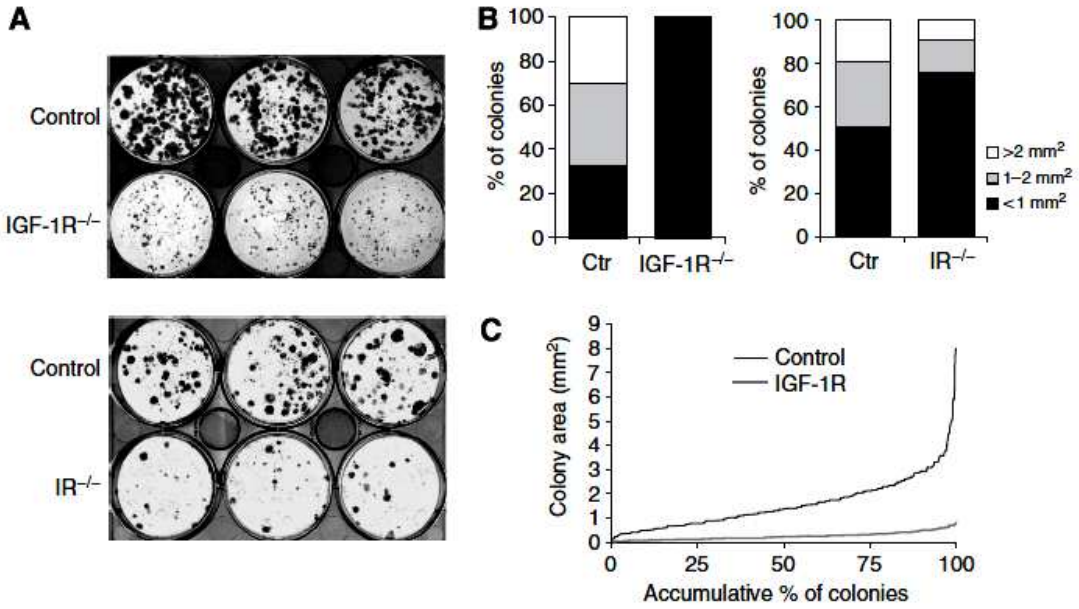

Figure 13: Reduced proliferative potential in the absence of IGF-1R/IR signaling.
(A). Colony forming assay using primary keratinocytes isolated from control, IR$^{-/-}$ and IGF-1R$^{-/-}$ mice. The results are shown for a representative experiment of three independent experiments done. (B) Quantification of the colony forming assays shown in (A). (C) Colonies of control (upper graph) versus IGF-1R-/- keratinocytes (lower graph) plotted as increasing colony size against accumulating percentage of colonies.

2.2 Insulin and IGF-1 receptors regulate the epidermal progenitor cell marker keratin15 (K15)

To examine whether alterations in proliferative potential were affecting epidermal progenitor cells, we used the progenitor cell marker keratin 15 (K15). Both, western blot analysis (Fig.14A) and immunofluorescent staining (Fig.14B) showed a dramatic reduction in the overall K15 protein levels in dko epidermis compared with control.

A recent study suggested that the colonies formed in the clonogenic assay are derived from the HF stem cells (Langton et al, 2008). Although this study used number, and not size, of colonies as a read-out this suggests IR/IGF-1R may exert

their effect on proliferative potential by affecting HF stem cells. Unlike IFE progenitor cells, HF stem cells are well characterized at the molecular level (Morris et al. 2004; Tumbar et al. 2004). Indeed, K15 is commonly used as a marker for hair follicle stem cells, although in newborn mice K15 is also expressed in basal interfollicular keratinocytes (Liu et al, 2003). We therefore assessed the epidermal compartments seperatly for K15 expression. A strong decrease in K15 staining was observed in IR-negative IFE compared to hair follicles (Fig.14C). In the absence of IGF-1R or in the dko mice K15 was strongly reduced in both compartments (Fig.14C) and was also more pronounced in the IFE compared to hair follicles (Fig.14C). This suggested that IR mainly regulates the interfollicular compartment, whereas IGF-1R signaling regulates both progenitor cell compartments.

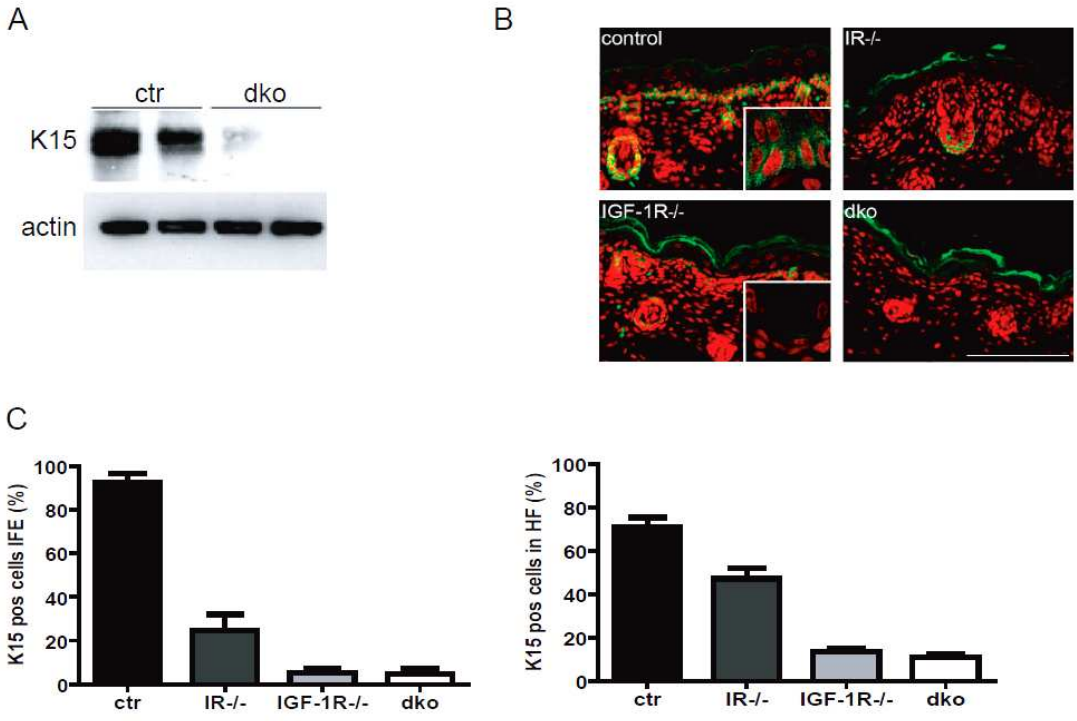

Figure 14: Reduced expression of the epidermal progenitor cell marker K15.

(A) Western blot analysis for keratin15 (K15) using lysates isolated from epidermis of newborn mice. An equal volume of lysates were run on a separate gel and probed for actin to control for loading. (B) Keratin 15 (green) staining on back skin of newborn mice. Nuclei were counterstained using propidium iodide (red). Scale bar is 100 µM. Inset shows high magnification of basal cell layer. (C&D) (C) Quantification of K15 positive basal IFE cells and HF in a 500 µM area of control, $IR^{-/-}$, $IGF\text{-}1R^{-/-}$ and dko epidermis. N=4 independent mice/genotype. (5-6 sections of each mouse).

As assessed by quantitative Real Time PCR analysis, other general epidermal proliferation and progenitor cell markers, such as Igfbp-5 (Blanpain, Lowry et al. 2004; Schlake 2005), Foxo1 and also K15 were reduced in dko epidermis compared to control (Fig.15), providing further evidence that insulin and IGF-1 receptor signaling regulates the epidermal progenitor cell compartment. Nevertheless, several other well known HF specific stem cell markers such as CD34, Tcf4, CdKn1b (Blanpain, Lowry et al. 2004; Morris, Liu et al. 2004) were not changed in dko compared to control, suggesting that the HF stem cell compartment remains nearly unaffected (Fig.15).

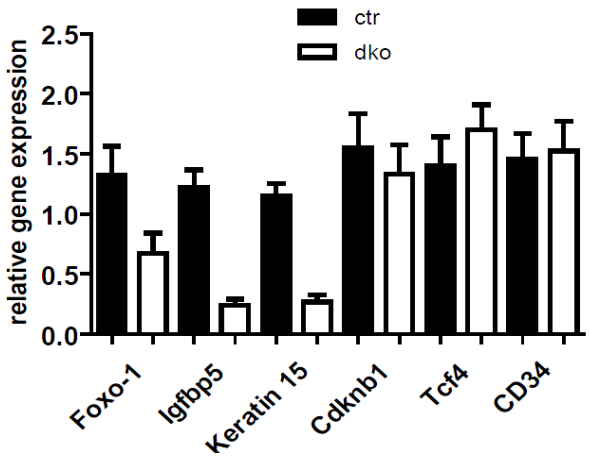

Figure 15: Reduced gene expression of progenitor cell markers but not HF stem cell markers.

Quantitative real time RT-PCR analysis on RNA isolated from newborn epidermis for the indicated markers. N=5 each for control and dko.

2.3 Reduction of label retaining cells in the IFE of IGF-1R$^{-/-}$ mice

Progenitor cells, due to their suggested slow cycling nature, are characterized by their ability to retain BrdU over prolonged periods after intra-periotenal (IP) injection (Braun, Niemann et al. 2003). As dko mice die within the first two days we assessed BrdU retention in IGF-1R$^{-/-}$ and control mice by injecting them for three consecutive days with BrdU and examining BrdU label retention after 15 days, 40 days and 70 days. Compared to controls, an 80% reduction in BrdU positive basal cells was found in the IFE of IGF-1R$^{-/-}$ mice after 15 days (Fig.16A) whereas a small but significant reduction was found in the number of BrdU positive cells in the hair follicles (Fig.16B).

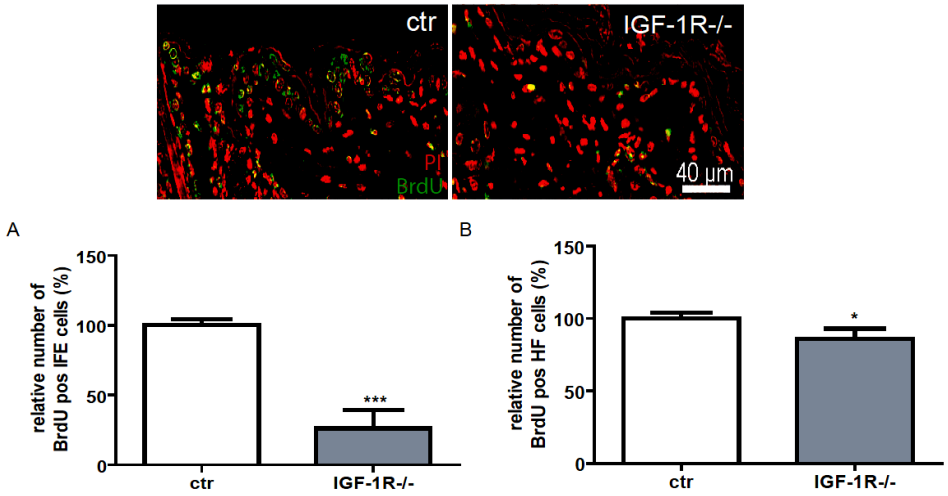

Figure 16: Loss of label retaining cells (15d) in the IFE of IGF-1R$^{-/-}$ mice.

Skin sections of epidermis 15d after BrdU labeling. BrdU (green) and nuclei were counterstained using propidium iodide (red). Scale bar is 40µM. (A) Quantification of BrdU positive IFE cells in 500 µm^2. (B) Quantification of BrdU pos HF cells. N= 3 mice/group; IFE: ***P < 0.0005; HF: *P<0.05 (5-6 sections per mouse).

The reduced number of label retaining cells was still observed after the 40 day chase period when the IFE has renewed at least once (Fig.17, upper panel). This was even more pronounced after 70 days of chase, where we could detect 60% reduction of BrdU positive cells in the IFE of IGF-1R$^{-/-}$ mice compared to control (Fig.17, lower panel).

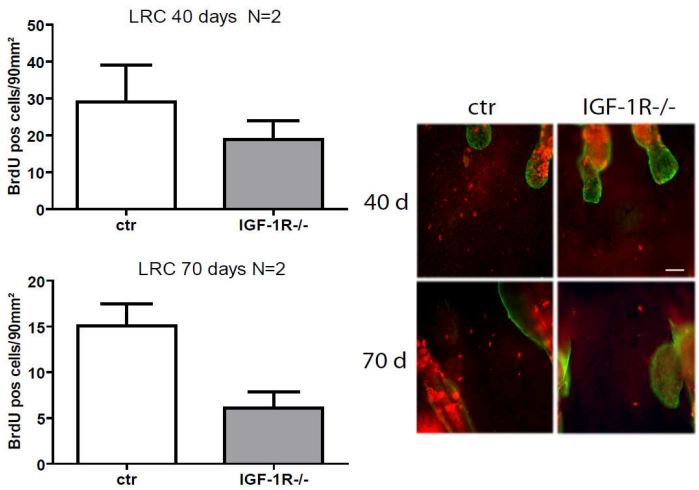

Figure 17: Loss of label retaining cells (40&70d) in the IFE of IGF-1R$^{-/-}$ mice.

Quantification in tail epidermis by whole mount staining of BrdU positive cells (red) in 90mm² of IFE in ctr and IGF-1R$^{-/-}$ mice at 40 days (upper panel) and 70 days (lower panel) after BrdU labeling. N = 2 mice/group; area: 90mm². Scale bar is 40µm

The loss of label retaining cells in the interfollicular epidermis, and the subsequent epidermal thinning could also result from an altered migration of the keratinocytes in the epidermis which would result in a shortened transition time (measurement of the transit time for cells through the epidermis). However, *in vitro* migratory properties of IGF-1R$^{-/-}$ keratinocytes were unchanged (not shown). Moreover, analysis of positive cells 24h after labeling with BrdU, a method previously used to assess differences in transition time (Reichelt and Magin 2002), did not reveal any difference either (not shown).

2.4 IR/IGF-1R is a key regulator of the small GTPase Rac1 in the epidermis

We next thought to identify how IR and IGF-1R could control proliferative potential of the interfollicular epidermis. Insulin and IGF-1R negatively affect the activity of Foxo transcription factors (Taniguchi, Emanuelli et al. 2006; Arden 2007), which have been implicated in stem cell regulation of the hematopoetic system (Tothova, Kollipara et al. 2007). A recent paper described a novel function of Foxo as co-activator of RBJ (Kitamura, Kitamura et al. 2007). Given the critical role of Notch in driving basal keratinocytes towards differentiation (Blanpain, Lowry et al. 2006), we decided to examine if impaired epidermal IR/IGF-1R signaling results in increased Notch activation thereby promoting differentiation concomitant with a decrease in proliferative potential. However, we were unable to detect any difference in either RNA or protein levels of the Notch target Hes1 (Fig.18A&B), nor did we observe any change in Hes1 nuclear localization (experiment done by Hady Ibrahim, not shown). In addition, as already shown in Figure 15 the total amount of Foxo1 RNA is decreased upon loss of IR and IGF-1R, which is possibly a consequence of the reduced number of progenitor cells. This suggests IR/IGF-1R does not regulate canonical Notch signaling in the epidermis.

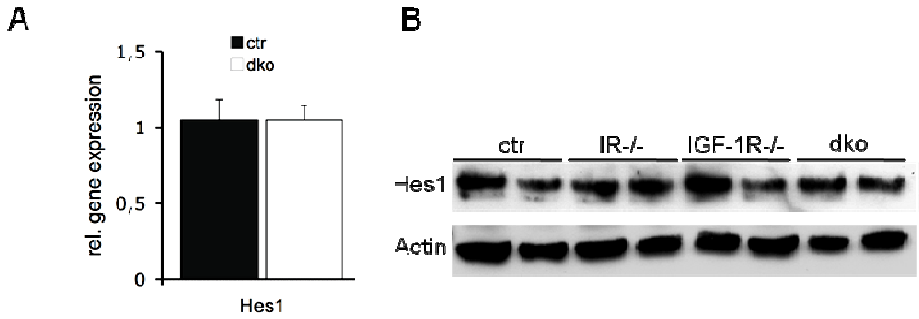

Figure 18: Notch pathway target Hes1 is not affected after IGF-1R deletion.
(A) Quantitative real time RT-PCR analysis of RNA isolated from newborn epidermis for Hes1. N=5 for both control and dko. (B) Western blot analysis for Hes1 on lysates isolated from epidermis of newborn mice. Same amount of lysates were run on a separate gel and probed for actin as a loading control.

Another potential candidate by which IR and IGF-1R signaling could determine proliferative potential of keratinocytes is by regulating the activity of the small GTPase Rac1 via activation of PI3-kinase (Welch, Coadwell et al. 2003). Rac1 has recently been implicated in the maintenance of epidermal stem cells (Benitah, Frye et al. 2005; Castilho, Squarize et al. 2007) but the upstream regulators in this process have not yet been identified. Analysis of Rac1 activity in IR- and IGF-1R-deficient skin revealed a dramatic 80% reduction of Rac1 activity compared to controls whereas total levels of Rac remained unaffected (Fig.19A&B).

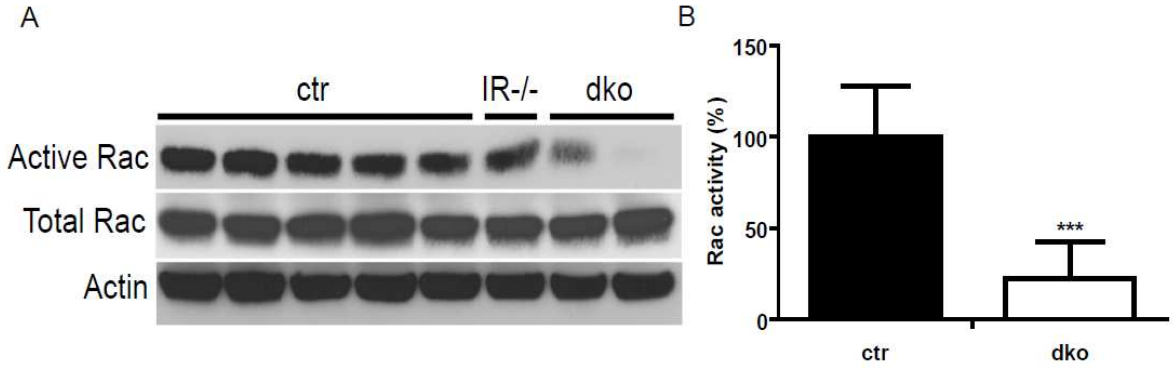

Figure 19: Insulin and IGF-1R signaling regulates epidermal Rac1 activity *in vivo*
(A) Western blot analysis of Rac1 activity assays in the epidermis of control, $IR^{-/-}$ and dko newborn mice. Shown is a representative experiment. (B) Quantification of Rac1 activity in the epidermis of control and dko newborn mice. N=9 for control and N= 5 for dko mice; ***P:0.0001.

We then performed further *in vitro* studies, which show Rac1 activation is directly dependant on insulin and IGF-1 stimulation in primary mouse keratinocytes (Fig.20A). Since Rac1 activity can regulate c-Myc (Benitah, Frye et al. 2005), which itself negatively controls the number of progenitor cells in the interfollicular epidermis (Arnold and Watt 2001; Waikel, Kawachi et al. 2001), we assessed if c-Myc levels were altered in the absence of IR and IGF-1R. In fact both protein and RNA levels of c-Myc were drastically increased in the epidermis of $IGF\text{-}1R^{-/-}$ and dko mice when compared to control (Fig.20B). A similar increase in c-Myc levels was observed in

primary dko keratinocytes (not shown). These results suggest that decreased Rac1 activation results in increased c-Myc activity in the dko mice.

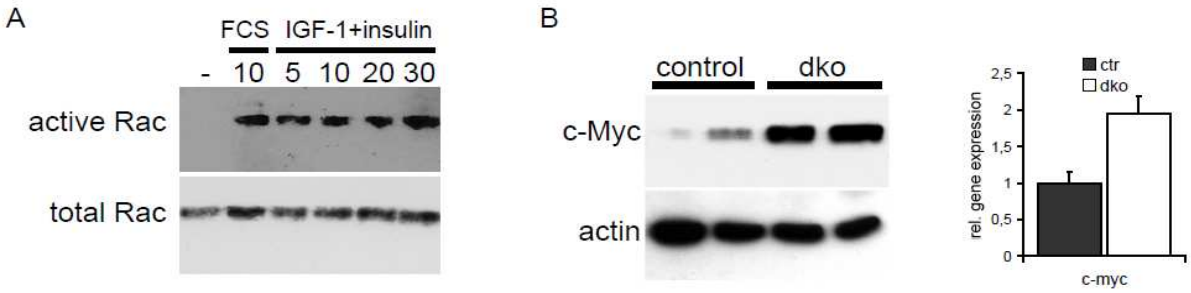

Figure 20: Insulin and IGF-1 signaling regulates epidermal Rac activity *in vitro* via c-Myc
(A) Insulin and IGF-1 directly activate the small GTPase Rac in mouse keratinocytes after serum starvation. Lane numbers refer to stimulation time in minutes. (B) Western blot and quantitative real time RT-PCR analysis of c-Myc in protein lysates and RNA isolated from newborn epidermis (N=5 for both control and dko). An equal volume of lysate was run on a separate gel and probed for actin.

2.5 IR/IGF-1R regulate proliferative potential via Rac1 in cultured keratinocytes

To address the question of whether IR/IGF-1R dependent activation of Rac1 is indeed important for regulation of proliferative potential of keratinocytes, we assessed if constitutive active Rac (RacDA) is able to increase proliferative potential in the absence of IR and IGF-1R. Therefore we are transfected dko keratinocytes with both GFP and RacDA lentivirus. Importantly, lentiviral expression of GFP itself did not impair but actually slightly enhanced colony forming capacity in control cells (Stachelscheid, Ibrahim et al. 2008).

After transduction of dko keratinocytes with either GFP or RacDA lentivirus the colony forming ability was assessed. Expression of RacDA in dko keratinocytes increased colony size when compared to GFP expression (Fig.21A&B). As RacDA may also directly affect the actin cytoskeleton, and therefore cell size, we wanted to rule out that the increase in colony size is a result of an increased cell size. The average number of cells/colony was significantly increased in RacDA compared to GFP expression cells (Fig.21C), thus showing that the larger colony size did not result from an enlarged cell size but was also due to increased proliferative potential.

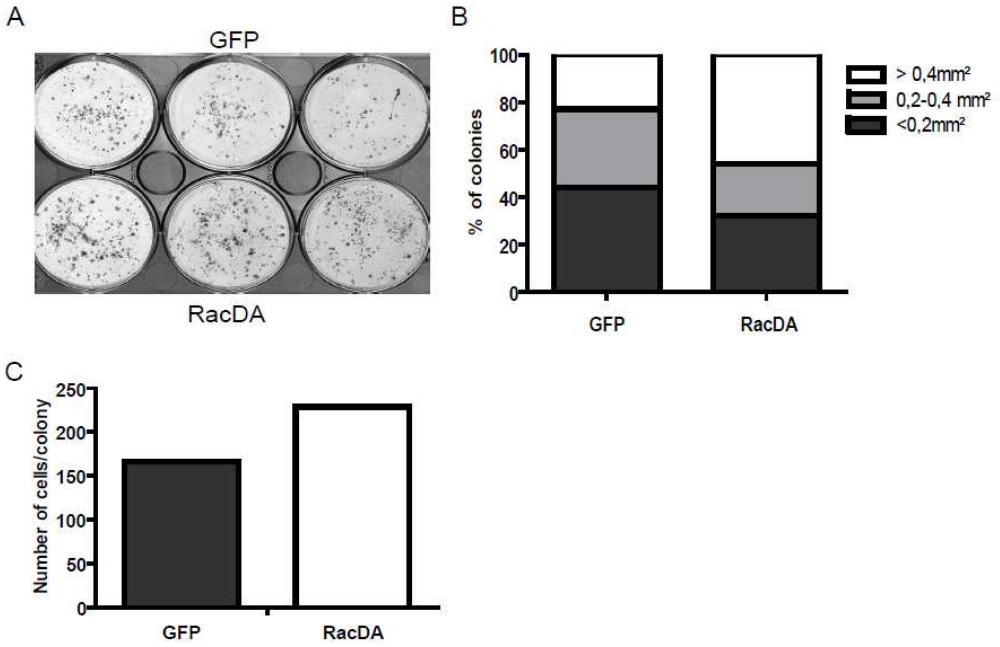

Figure 21: Epidermal insulin/IGF-1R determine the proliferative potential of progenitor cells *in vitro* via activation of the small GTPase Rac.

(A) Colony forming assay of dko primary keratinocytes transduced with either GFP- or RacDA lentivirus and (B) quantification of colony size of the experiment shown in A. (C) The average number of cells/colony in RacDA compared to GFP expression cells **P<0.001. N=3

2.6 Rac1 activation is necessary for epidermal morphogenesis in IGF-1R$^{-/-}$ mice

If Rac1 dependent regulation of proliferative potential by IGF-1R/IR is relevant for *in vivo* epidermal morphogenesis, *in vivo* expression of RacDA in the epidermis should restore the hypomorphic epidermis induced by loss of IR and/or IGF-1R. To directly test this hypothesis we crossbreed mice with epidermal deletion of IGF1-R with mice showing a K14 mediated basal expression of a myc-tagged RacDA (IGF-1R$^{-/-}$; RacDA, Fig.22A western blot, see material and methods for crossings) and compared the thickness of newborn epidermis and palate with those of IGF-1R$^{-/-}$ littermates. Expression of RacDA significantly ameliorated the hypomorphic epidermis caused by absence of IGF-1R (Fig.22A&B). The reversal is not complete since the thickness of the epidermis, showing a K14 mediated basal expression of a myc-tagged RacDA, is not similar to ctr epidermis. Nevertheless, RacDA was more efficient, even though not complete, rescuing the thinner palate in IGF-1R$^{-/-}$ mice (Fig.22C).

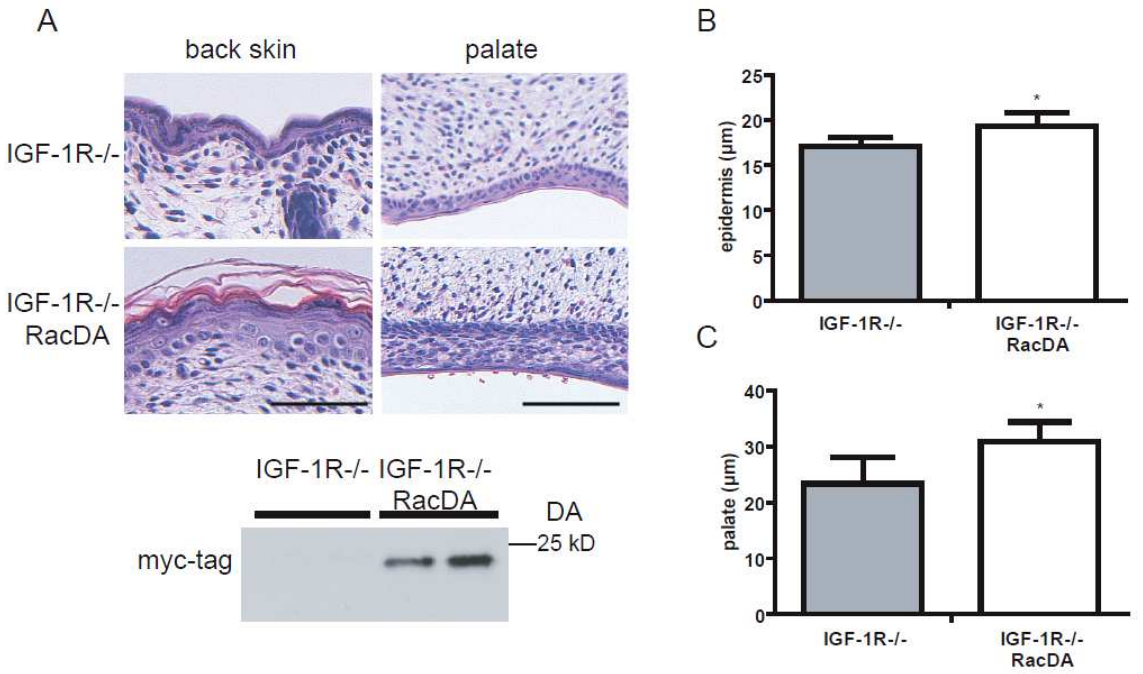

Figure 22: Rac activation is necessary for epidermal morphogenesis in IGF-1R$^{-/-}$ mice.
(A) H&E staining of back skin (scale bar is 50 µM) and palate (scale bar is 100 µM) of IGF-1R$^{-/-}$ and IGF-1R$^{-/-}$ RacDA mice. Lower panel: western blot analysis of myc-tagged RacDA in newborn IGF-1R$^{-/-}$ and IGF-1R$^{-/-;}$RacDA mice (B &C) Quantification of the thickness of the epidermis (B) (without stratum corneum) and palate (C) in IGF-1R$^{-/-}$ and IGF-1R$^{-/-}$;RacDA mice. N=5 mice/genotype. P* = 0.02.

We propose a working model, shown in Figure 23, showing that epidermal IR and IGF-1R are crucial regulators of the small GTPase Rac1. Rac1 is a small (~21 kDa) signaling protein and belongs to the Rho family of GTPases. All regulatory GTPases have a common mechanism that enables them to switch from the active GTP-form to the inactive GDP-form. We suggest that IGF-1R/IR may regulate the switch from inactive Rac^{GDP} to the active Rac^{GTP} form by determining proliferative potential and IFE morphogenesis in newborn epidermis. If and how proliferative potential regulates epidermal morphogenesis remains an open question, especially considering the unaltered proliferation we observed in newborn mice (see introduction). We propose that IGF-1R/IR signaling regulates proliferative potential during embryonic development. Furthermore, the hypomorphic epidermis seen in newborn skin after IGF-1R/IR deletion could derive due to cell cycle arrest, delay or senescence during epidermal development.

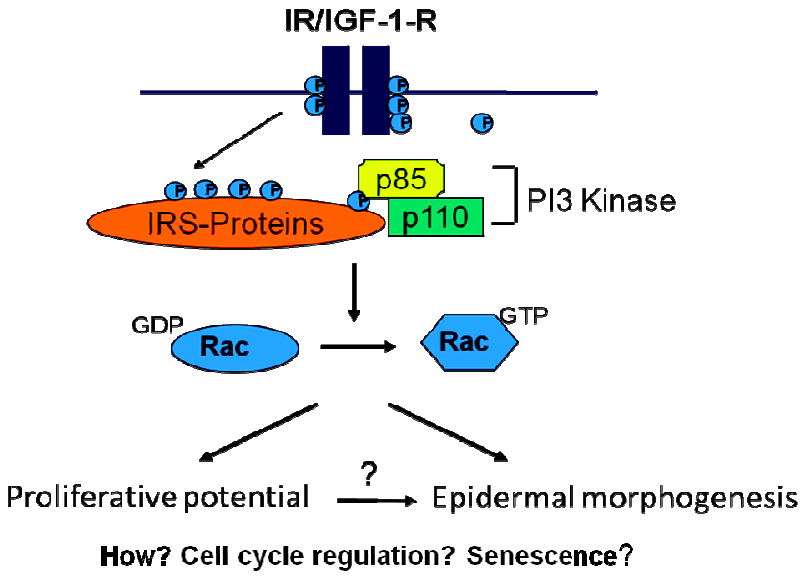

Figure 23: Rac1 as a key target of the epidermal IR/IGF-1R pathway.

The small GTPase Rac1 is a key target of epidermal IR/IGF-1R signaling crucial for proliferative potential and epidermal interfollicular morphogenesis. A possible mechanism how proliferative potential regulates epidermal morphogenesis is through the cell cycle and/or senescence.

2.7 IGF-1R but not IR regulates epidermal proliferation during embryonic development and in primary keratinocytes

To determine at which developmental stage loss of IR and/or IGF-1R affects epidermal morphogenesis and whether this relates to temporary changes in proliferation different stages of embryonic development were examined. Since the phenotype is most prominent in dko epidermis we focused on these embryos. E15.5 dko mice showed the expected 2-3 epidermal layers; indistinguishable from control mice (Fig.24). In dko mice first indications of a hypomorphic epidermis became apparent at E16.5. At this time point control mice have a 4-6 layer epidermis while the dko epidermis only showed 3-4 layers, closer resembling E15.5 epidermis (Fig.24).

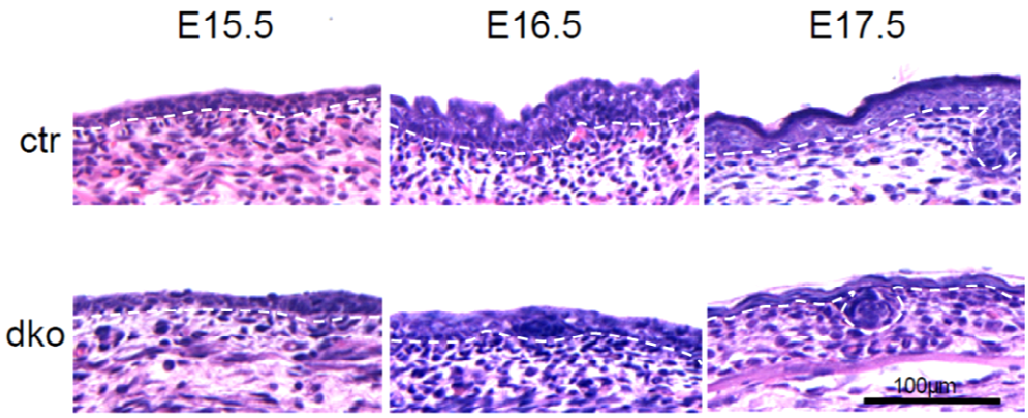

Figure 24: Hypomorphic epidermis prenatal at E16.5 in dko embryos.
H&E stainings of E15.5, E16.5 and E17.5 ctr and dko back skin. The white line represents the basement membrane which separates the epidermis from the dermis. Epidermis is above the white line. Scale bar is 100µm. N=3 from all genotypes.

To examine if the reduced number of layers first observed at E16.5 in dko mice was due to changes in proliferation or apoptosis we stained for the proliferation marker Ki67 and performed Tunel assays on different developmental stages. Surprisingly, no changes in either Ki67 (Fig.25) or Tunel staining (not shown) were found at E15.5 and E16.5. This indicates that the inability to increase the number of suprabasal layers in the absence of IR/IGF-1R at E16.5 is not likely to be due to changes in apoptosis and may not be as a result of altered proliferation. At E17.5, both control and dko mice showed continuing epidermal stratification, even though the dko epidermis remained hypomorphic (Fig.24). Surprisingly, this coincided with reduced Ki67 staining indicating a reduction in proliferation (Fig.25). No change in Tunel staining was observed, indicating that apoptosis was similar between ctr and dko E17.5 epidermis (not shown). E17.5 $IR^{-/-}$ embryos, which exhibit the mildest phenotype, showed no obvious change in proliferation even though the epidermis was hypomorphic (data not shown).

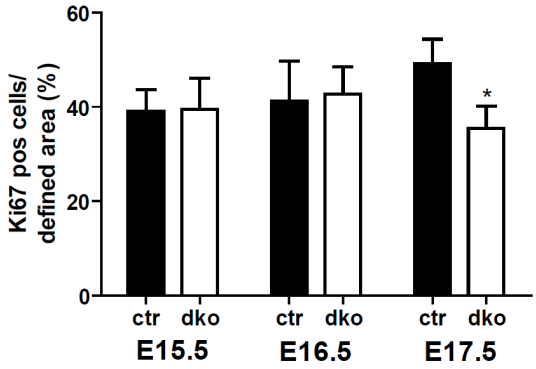

Figure 25: IGF-1R/IR signaling regulates proliferation during embryonic development.
Quantification of Ki67 positive cells in epidermal basal cells of E15.5, E16.5 and E17.5 embryos. N=3 for each genotype $p>0.05$ for E15.5 and 16.5, $P<0.01$ for E17.5.

2.8 IGF-1R plays a more prominent role in the regulation of mitosis

Ki67 staining was not reduced in E16.5 old dko embryos even though this is the first stage the phenotype is macroscopically visible. Since Ki67 is expressed during all cell cycle stages, this result does not rule out an arrest in either G2/M or in mitosis. To directly test this, the number of anaphase spindles was counted in E16.5 epidermis. If cells undergo an arrest at G2/M or in mitosis, the prediction would be a reduction in the number of cells with anaphase spindles. Indeed a dramatic loss of anaphase spindles was observed in both dko as well as IGF-1R$^{-/-}$ E16.5 epidermis (Fig.26). In addition a slight, although non-significant, reduction was also seen in the epidermis of E16.5 IR$^{-/-}$ mice, in agreement with the fact that these mice showed only a mild phenotype compared to control.

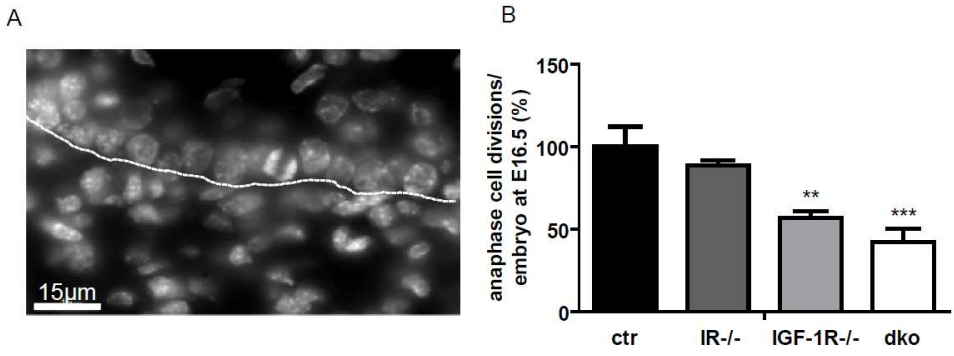

Figure 26: G2/M or mitotic spindle block *in vivo* in IGF-1R$^{-/-}$ and dko E16.5 embryonic epidermis.
(A) Anaphase cell division in the basal layer of E16.5 ctr epidermis. Scale bar is 15µm.
(B) Quantification of anaphase cell divisions in the epidermal basal layer and at different levels of the epidermis in ctr, IR-/-, IGF-1R-/- and dko embryos (E16.5). N=3 of each genotype.

A reduction in the number of cells in anaphase may indicate cells are arrested at G2/M or that they are undergoing mitotic arrest, also known as a spindle checkpoint arrest. The spindle checkpoint takes place between the metaphase and anaphase stages of mitosis. To distinguish between a block at G2/M or mitosis we counted the number of metaphase spindles at E16.5 and compared this to the number of anaphase spindles. If cells arrest at the G2/M transition, we would expect to observe a reduction in both the number of cells with metaphase and anaphase spindles. On the other hand, if cells undergo a mitotic spindle block, more cells with metaphase would be observed at E16.5 in dko versus control. An increased number of cells with metaphase spindles were indeed observed in dko E16.5 embryos when compared to control (Fig.27). This strongly suggested that upon loss of epidermal IGF-1R/IR, basal keratinocytes at E16.5 arrest at the spindle checkpoint.

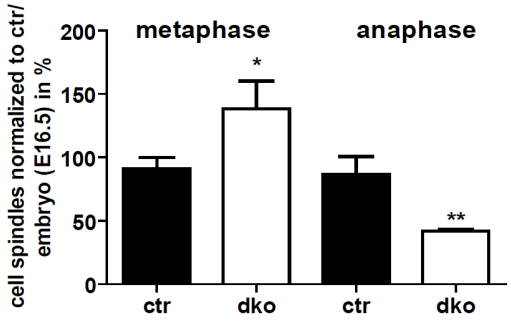

Figure 27: Mitotic spindle block *in vivo* in dko E16.5 embryos.

Quantification of metaphase and anaphase cell divisions in the epidermal basal layer of ctr, and dko E16.5 embryos. Different levels of the epidermis within the embryos were counted. Quantification was done by normalizing ctr spindles to 100%. N=3 of each genotype.

To examine if the mitotic spindle block only occurs in the context of the embryo, primary IGF-1R$^{-/-}$ and control keratinocytes from newborn mice were isolated. *In vitro* cell cycle analysis by FACS confirmed an increased amount of 4n DNA content which represents G2/M cells and also an increased number of so-called 8n DNA content cells in IGF-1R$^{-/-}$ keratinocytes (Fig.28A&B). 8n cells show the double amount of DNA compared to G2/M cells, and probably arise due to incomplete division after chromosome duplication. These cells are not yet well characterized but some groups have classified them into aneuploid cells after chromosome missegregation (Sunavala-Dossabhoy, Li et al. 2003).

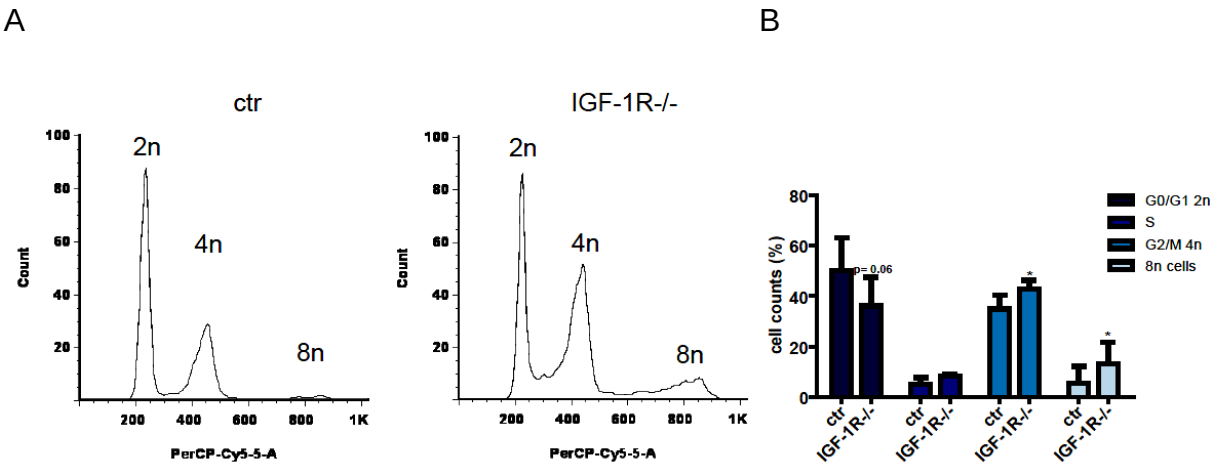

Figure 28: G2/M block *in vitro* in IGF-1R-/- primary keratinocytes.

(A) FACS analysis of the cell cycle in ctr and IGF-1R-/- fixed keratinocytes (P3). 2n, 4n and 8n represent DNA content of the cells, measured by PI. The results shown are a representative of three independent experiments. Diagram was designed using the Flow Jo software. (B) Quantification of 2n, 4n and 8n DNA content in ctr and IGF-1R-/- keratinocytes. N=3 of each genotype and with the same passages (P2&3).

Furthermore, similar to E16.5 epidermis, an increase in the number of metaphase cells was observed in IGF-1R$^{-/-}$ versus control cultered primary keratinocytes, whereas the number of anaphase cells was reduced (Fig.29B). Thus, loss of IGF-1R also induces a spindle block when cells are in culture. These findings strongly suggest a requirement for IGF-1R signaling to properly progress through mitosis.

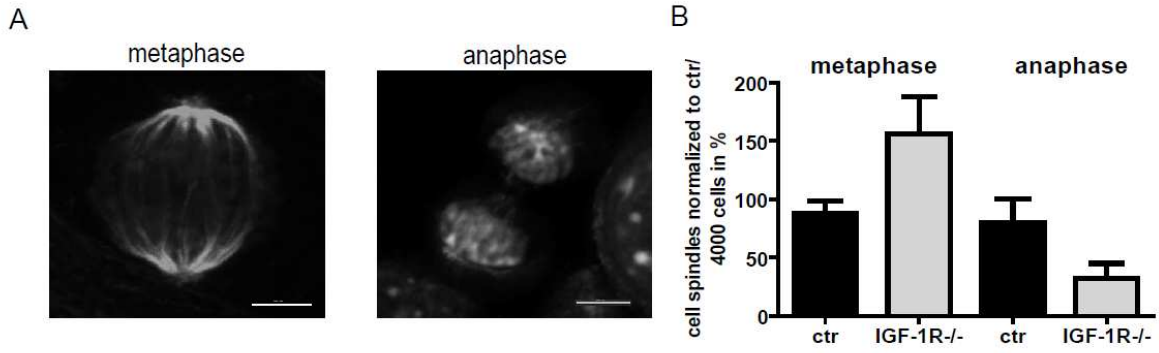

Figure 29: Mitotic spindle block *in vitro* in IGF-1R-/- primary keratinocytes.

(A) Representative metaphase and anaphase cell division in primary ctr keratinocytes. Scale bar is 15µm. (B) Quantification of metaphase and anaphase cell divisions in primary ctr and IGF-1R-/- keratinocytes. Quantification was done by normalizing ctr spindles to 100%. N=3 for each genotype.

2.9 Altered gene and protein expression of important cell cycle regulators

To get an overview of possible differentially regulated cell cycle genes implicated in G2/M or mitosis progression we performed global expression analysis (not shown) on RNA using affymetrix microarrays from control and dko epidermis of newborn mice. This analysis revealed that several genes implicated in the regulation of cell cycle progression, such as Mdm2, 14-3-3sigma and cyclin G1, were significantly upregulated in dko newborn epidermis compared to controls. These results were confirmed by real time PCR analysis on an independent set of isolated epidermal RNAs (Fig.30A). Furthermore, western blot analysis showed an increased amount of phosphorylated p38 (P-p38) MAPKinase, indicative of p38 activation, as well as an increase in 14-3-3σ protein, a downstream mediator of p38 MAPK in dko newborn epidermis compared to control (Fig.30B).

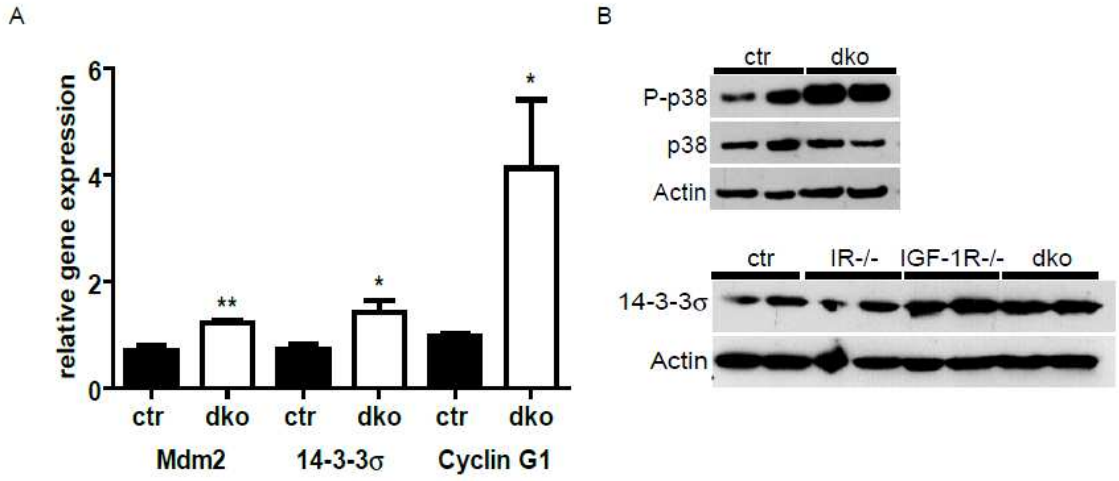

Figure 30: Cell cycle genes and protein expression altered in IGF-1R-/- and dko newborn epidermis

(A) Quantitative real time RT-PCR analysis of RNA isolated from newborn epidermis for the indicated markers. N=4 for both control and dko (B) Western blot analysis for MAPK p38 and 14-3-3σ on protein lysates isolated from newborn ctr, IR-/-, IGF-1R-/- and dko mice. Equal volumes of lysate were run on a separate gel and probed for actin as a loading control.

To test whether increased p38 activity might be the reason for the hypomorphic epidermis seen at E16.5 dko embryos (Fig.24) we performed immunofluorescence stainings of P-p38 in the epidermis of E16.5 (Fig.31A) embryos and newborn mice (Fig.31B). In newborn epidermis we could detect areas in the dkos which show a high level of P-p38 expression in all epidermal layers, in line with the previously shown

western blot analysis, but no obvious differences between ctr and dko epidermis at E16.5 could be observed (Fig.31A).

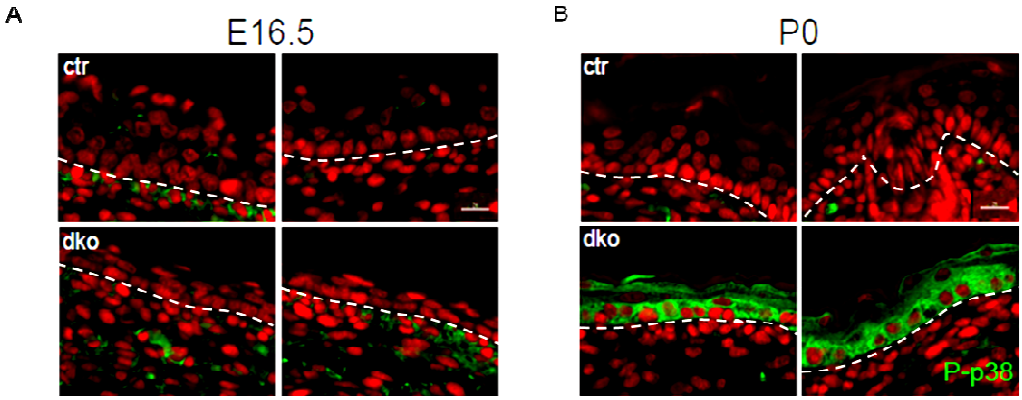

Figure 31: MAPK kinase p38 is not responsible for the hypomorphic epidermis in dko E16.5 embryos.

Immunofluorescence staining of P-p38 (green) in ctr and dko E16.5 and newborn (P0) epidermis. Nuclei were counterstained by propidium iodide (red). Scale bar is 20µm. White line should represent the basement membrane separating epidermis and dermis. N=2

Thus, the result suggests that stress kinase p38 activity is not responsible for the hypomorphic epidermis in E16.5 dko embryos and therefore is not a direct target of the IGF-1R signaling pathway. An indirect effect, e.g. stress activity during birth, may cause the increased in kinase activity.

Interestingly, three of the deregulated genes in Fig.30, MDM2, Cyclin G1 and 14-3-3σ, have been implicated in G2/M arrest and cooperate with the tumor suppressor p53, a major regulator of the cell cycle (Alarcon-Vargas and Ronai 2002) (Kimura, Ikawa et al. 2001) (Fornari, Gramantieri et al. 2009) (Chan, Hermeking et al. 1999). Therefore we analyzed p53 expression in ctr and IGF-1R/IR knockout epidermis and identified increased p53 activity in IGF-1R-/- and dko newborn epidermis (Fig.32).

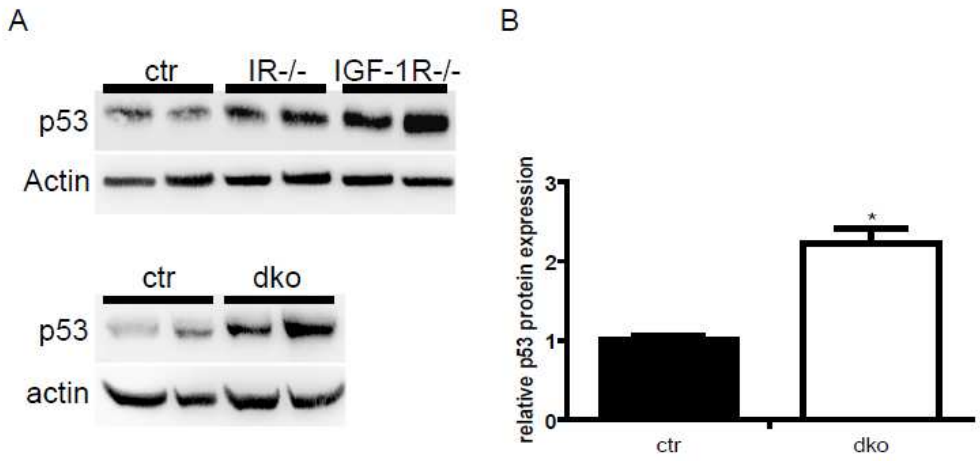

Figure 32: Increased p53 protein expression after IGF-1R/IR deletion

(A) Western blot analysis of p53 on lysates isolated from newborn ctr, $IR^{-/-}$, $IGF\text{-}1R^{-/-}$ and dko mice. Equal volumes of lysates were run on a separate gel and probed for actin as a loading control. (B) Quantification of western blot analysis shown in A of relative p53 protein expression in ctr and dko newborn epidermis. N= 4 ctr, dko and 2 $IGF\text{-}1R^{-/-}$, $IR^{-/-}$ epidermal lysates

Because the hypomorphic epidermis occurred earlier in development than at birth, such as was analyzed here, it remains unclear if increased p53 activity is a cause or consequence of the phenotype.

2.10 Loss of asymmetric cell divisions in $IGF\text{-}1R^{-/-}$ and dko E16.5 embryos

One explanation for the hypomorphic epidermis, in the absence of IGF-1R and/or IR, could be disturbed stratification during embryonic development. Lechler et al. suggested that the formation of suprabasal layers in the epidermis occurs through asymmetric cell divisions, in which the mitotic spindle orients perpendicularly to the basement membrane resulting in one basal daughter cells and one suprabasal daughter cell. To examine if a reduction in the number of asymmetric divisions may explain the reduction in suprabasal layers first obviously observed at E16.5, we re-analyzed the anaphase spindles in dko E16.5 epidermis and counted how many of those were asymmetric versus symmetric compared to control (Fig.33A). A similar analysis was performed on E16.5 $IR^{-/-}$ and $IGF\text{-}1R^{-/-}$ embryos. As shown in Fig.33B, quantification of E16.5 $IR^{-/-}$ epidermis showed no difference in symmetric cell divisions and only a slight decrease of asymmetric cell divisions compared to control. In contrast E16.5 $IGF\text{-}1R^{-/-}$ and dko epidermis showed a strong reduction in asymmetric cell divisions compared to control whereas symmetric cell divisions were

reduced but to a much lesser extent. These results are in line with our *in vivo* results showing that loss of epidermal IR affects epidermal thickness less severely than the loss of IGF-1R signaling (see introduction).

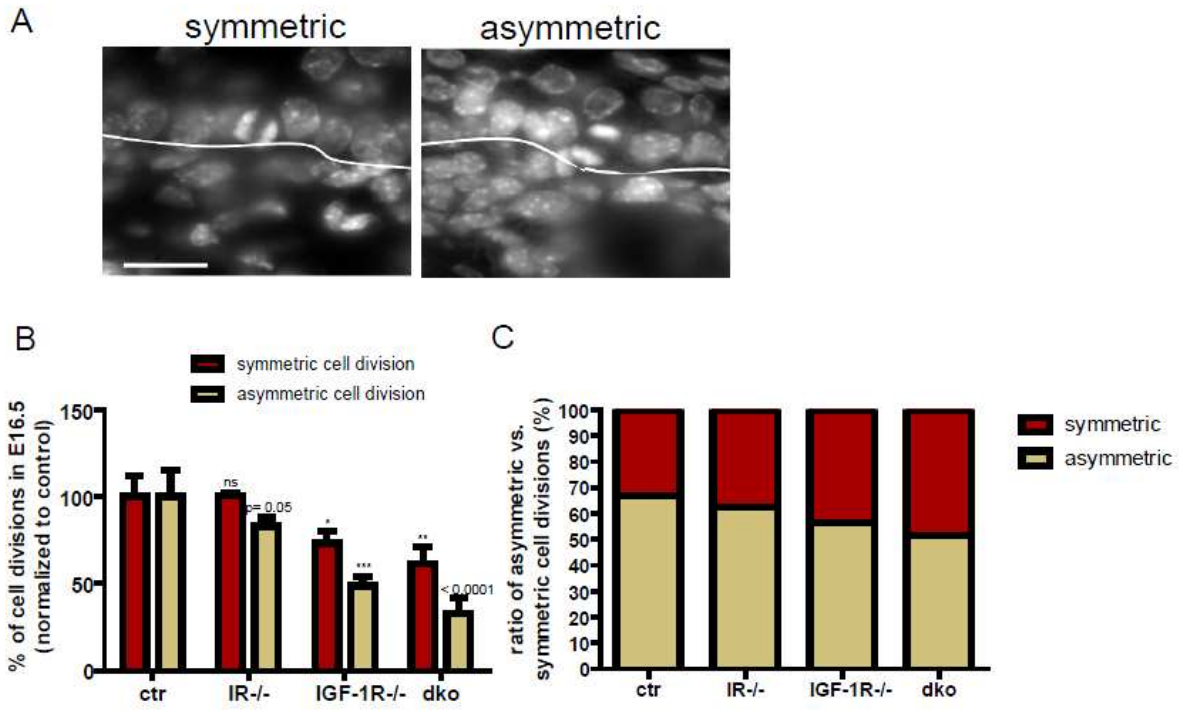

Figure 33: Loss of asymmetric cell division *in vivo* in IGF-1R$^{-/-}$ and dko E16.5 embryos.

(A) Symmetric (parallel spindle orientation to the basement membrane) and asymmetric (perpendicular spindle orientation to the basement membrane) cell divisions in the basal layer of E16.5 ctr epidermis. White line represents the basement membrane separated epidermis and dermis. Scale bar is 15µm. (B) Quantification of symmetric and asymmetric cell divisions in the epidermal basal layer at different levels of ctr, IR-/-, IGF-1R-/- and dko embryos (E16.5). Control symmetric and asymmetric cell divisions were normalized to 100%. N=3 of each genotype. (C) Ratio of asymmetric/symmetric cell divisions in the basal layer of E16.5 ctr, IR-/-, IGF-1R-/- and dko epidermis. Control anaphase cell divisions (asymmetric& symmetric cell divisions) were set to 100%.

In E16.5 control epidermis approximately 70% of anaphase spindles are asymmetric whereas only 30% are symmetric (Fig.33C), similar to what was published previously (Lechler and Fuchs 2005). Surprisingly, in IGF-1R$^{-/-}$ and dko E16.5 embryos the ratio of asymmetric/symmetric shifted from around 70%/30% to around 50%/50%

(Fig.33C), which may explain the reduced number of suprabasal layers in IGF-1R$^{-/-}$ and dko E16.5 epidermis.

2.11 Decreased p63 expression in basal keratinocytes after IGF-1R/IR deletion

As shown in Figure 13 the deletion of IGF-1R and IR results in less proliferative potential *in vitro*. Recently, it was described that proliferative potential could be associated *in vivo* with the transcription factor p63 (Senoo, Pinto et al. 2007) which is expressed in stem cells of multilayered squamous epithelia and acts as a key regulator of proliferation and differentiation (Blanpain and Fuchs 2007; Candi, Rufini et al. 2007; Senoo, Pinto et al. 2007). The main function and upstream regulators of p63, especially in the epidermis, are not well understood. To study the role of p63 in epidermal IGF-1R signaling, we performed immunofluorescence stainings of p63 in ctr and dko newborn epidermis. Indeed, a reduced number of p63 expressing cells were detected in the epidermal basal layer in newborn dko compared to control mice. This confirms our *in vitro* studies that IGF-1R signaling regulates proliferative potential (Fig.13). More interestingly, in newborn control epidermis almost every basal cell in the interfollicular epidermis (IFE) expressed p63 whereas newborn dko IFE revealed atypical areas of basal cells which have lost p63 expression (Fig.34A). Again this may explain our *in vitro* data suggesting IGF-1R/IR$^{-/-}$ keratinocytes have lost a cell population with high proliferative potential.

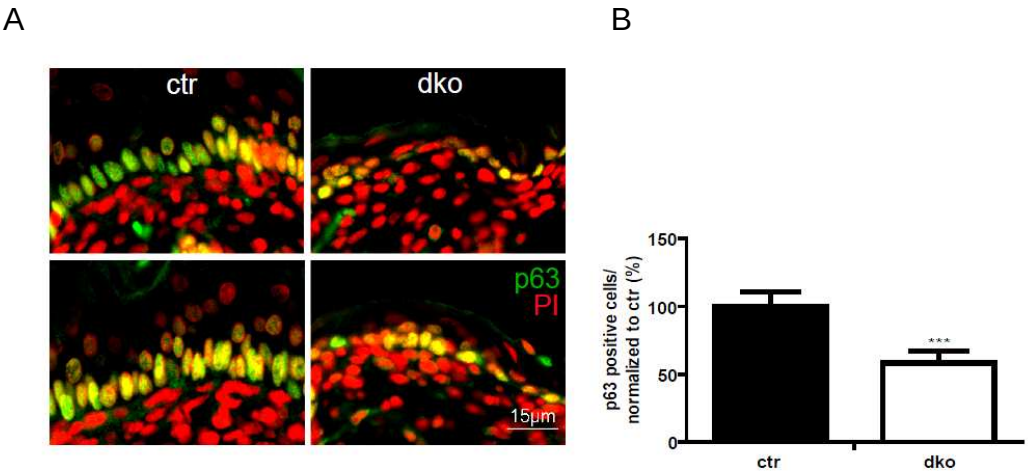

Figure 34: Missing p63 expression in newborn dko epidermis

(A) Immunofluorescence staining of p63 (green) on paraffin sections of newborn epidermis. Nuclei were counterstained using propidium iodide (red). Scale bar is 15 µM. (B) Quantification of epidermal basal p63 positive cells. The number of p63 positive cells were normalized to ctr to 100%. N=3 of each genotype

Furthermore, the basal layer of p63$^{-/-}$ mice shows a similar disturbed stratification with an almost complete loss of asymmetric cell divisions also at E16.5, suggesting a role of p63 in the stratification process during embryonic development (Lechler and Fuchs 2005). Although the epidermal phenotype in p63$^{-/-}$ mice is much more severe than the observed phenotype in IGF-1R/IR$^{-/-}$ epidermis, p63 may be an interesting downstream mediator of IGF-1R/IR signaling coordinating epidermal stratification via asymmetric cell division. Furthermore the residual p63 expression in IGF-1R/IR$^{-/-}$ newborn mice could explain the milder phenotype (see Fig. 34). To further address the question if p63 mediates IGF-1R/IR signal regulation of epidermal stratification we investigated p63 expression in dkos during embryonic development as well, especially at E16.5 where the hypomorphic epidermis first occurred.

Close investigation of the expression pattern of p63 in the basal layer shows, although the phenotype is much milder than in dko newborn mice, some localized areas with reduced or even absent p63 expression in basal dko cells (Fig.35, white arrow). In conclusion, decreased p63 expression in dko mice may derive from either a generally reduced proliferative potential in all basal cells or a specific loss of interfollicular epidermal progenitor cells. Furthermore, p63 could network with IGF-1R signaling regulating precise spindle formation, via asymmetric/symmetric cell division, to increase the suprabasal layers in the epidermis during embryonic development.

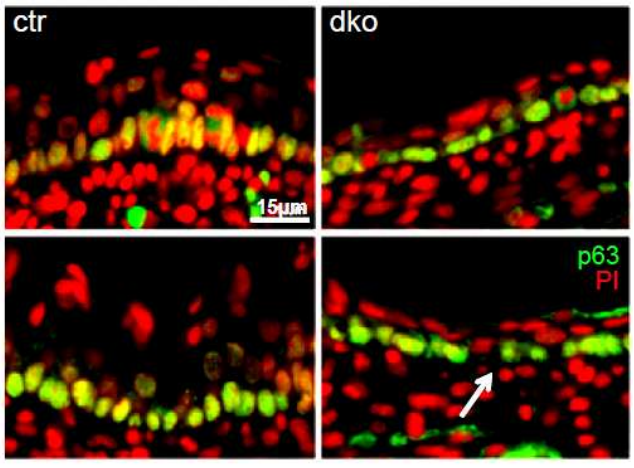

Figure 35: Missing p63 expression in E16.5 dko basal cells.

Immunofluorescence staining of p63 (green) on paraffin sections of E16.5 ctr and dko epidermis. DNA was counterstained by PI (red). Scale bar is 15 µm. N=3 for each genotype

2.12 Disturbed spindle formation in IGF-1R$^{-/-}$ keratinocytes

An explanation for the spindle block and the loss of asymmetric cell division in IGF-1R/IR$^{-/-}$ epidermis could be a disturbed spindle formation during mitosis. The microtubule-organizing center (MTOC) is a structure found in eukaryotic cells from which microtubules (α and γ tubulin) emerge and enabling centrosome formation, the MTOC can be visualized in cells by immunohistochemical detection of γ-tubulin. To examine whether IGF-1R/IR deletion results in a failure of spindle formation, we analyzed spindle and centrosome formation in metaphase primary ctr and IGF-1R$^{-/-}$ keratinocytes visualized by α and γ-tubulin double staining. Interestingly, γ-tubulin expression seemed to be reduced in IGF-1R$^{-/-}$ keratinocytes (Fig.36A) and α-tubulin staining revealed an increased number of metaphase cells in IGF-1R$^{-/-}$ keratinocytes with additional atypical spindles (Fig.36A, lower right cell). Moreover, the cells showing an atypical spindle were significant increased in IGF-1R$^{-/-}$ keratinocytes compared to control (Fig.36B; IGF-1R$^{-/-}$ 20-30%; ctr 10%).

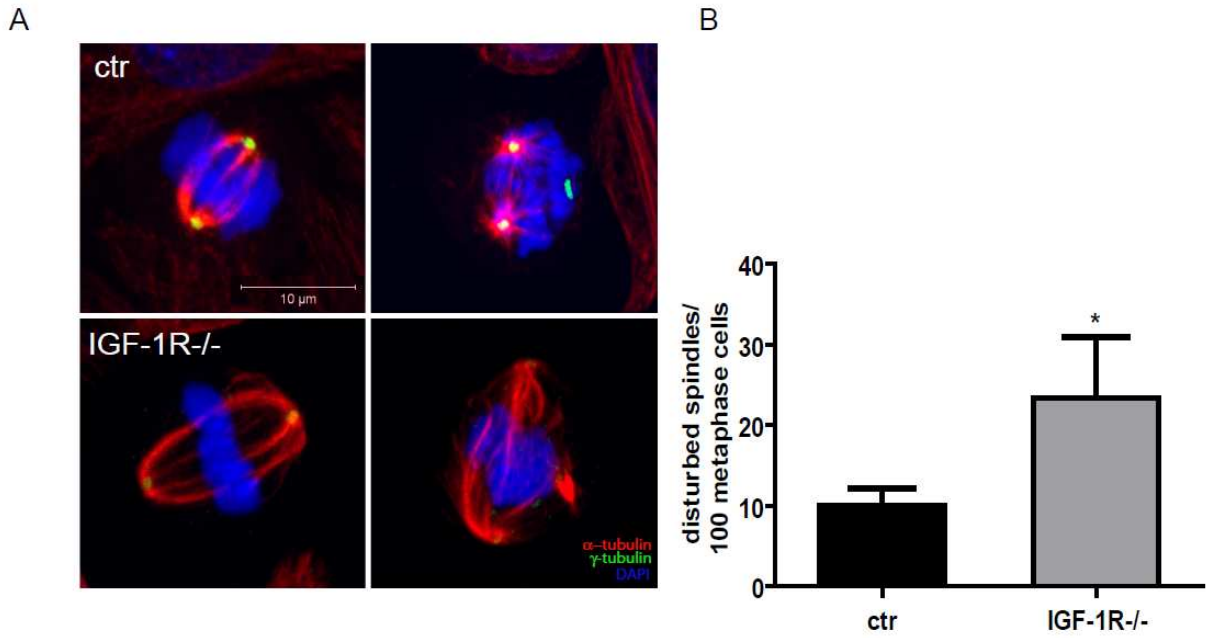

Figure 36: Disturbed spindle formation in IGF-1R-/- metaphase keratinocytes

(A) Immunofluorescence staining of α-tubulin (red) and γ-tubulin (green) in primary metaphase keratinocytes. DNA was counterstained by DAPI (blue). Scale bar is 10µm. White arrow represents atypical spindle in a representative metaphase cell (B) Quantification of the immunofluorescence staining by counting disturbed spindles/100 metaphase cells. N=3

2.13 Altered AuroraB expression in IGF-1R$^{-/-}$ keratinocytes and in E16.5 dko epidermis

The disturbed spindle formation observed in IGF-1R-/- metaphase spindles may be a cause of missegregated chromosome alignment during mitosis. One important family involved in these processes is the Aurora kinase family. In particular the isoforms AuroraA and B kinases are associated with microtubules during chromosome movement and segregation (Murata-Hori, Tatsuka et al. 2002). To analyze the activity levels of AuroraA and B in IGF-1R$^{-/-}$ keratinocytes, we examined AuroraA and B protein expression and localization particulary in ctr and IGF-1R$^{-/-}$ metaphase cell divisions. As expected in control metaphase cells AuroraA is co localized at the centrosomes and AuroraB is expressed at the equatorial plate, probably at the kinetochores (Fig.37). In IGF-1R$^{-/-}$ keratinocytes metaphase cells showed no differences in AuroraA localization. Interestingly, the ratio of IGF-1R$^{-/-}$ metaphase cells (20-30%) that showed disturbed centrosome and spindle formations presented in Figure 36 was similar to the increased number of centrosomes seen by AuroraA staining (Fig.37). Furthermore, AuroraB expression seems to be reduced or mislocalized in IGF-1R$^{-/-}$ metaphase cells showing disturbed spindle and centrosome formation (Fig.37).

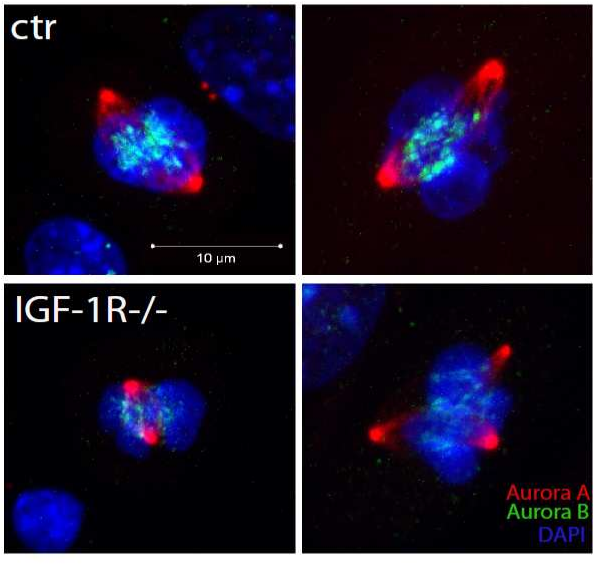

Figure 37: Mislocalized or reduced AuroraB expression in disturbed IGF-1R$^{-/-}$ metaphase spindles.

Immunofluorescence staining of AuroraA (red) and B (green) in ctr and IGF-1R$^{-/-}$ primary methanol fixed metaphase keratinocytes. DNA was counterstained by DAPI (blue). Scale bar is 10µm. N=3 of each genotype.

Additional western blot analysis did not reveal altered protein expression levels of total AuroraB in IGF-1R$^{-/-}$ keratinocytes (Fig.38). This could be explained by the low number of mitotic cells in the total cell lysate or by a mislocalization of AuroraB, without decreased AuroraB expression.

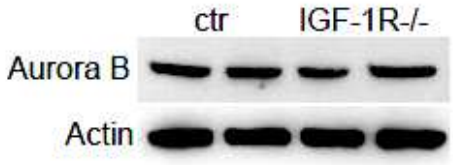

Figure 38: Total AuroraB protein levels are not altered in IGF-1R$^{-/-}$ keratinocytes.
Western blot analysis for total AuroraB on lysates isolated from ctr and IGF-1R-/- primary keratinocytes. Equal volumes of lysates were run on a separate gel and probed for actin as a loading control. N=3 of each genotype.

Furthermore, *in vivo* staining of AuroraB in E16.5 control epidermis showed clear expression of AuroraB in metaphase cells close to kinetochores and in anaphase cells at the central spindle in the basal layer of the epidermis (Fig.39, white arrows). Unlike E16.5 dko epidermis where AuroraB seems to be mislocalized or reduced in basal metaphase and anaphase cell divisions (Fig.39, white arrows). Only metaphase cells lying close to hair follicles show clear Aurora B expression close to kinetochores (Fig.39, red arrow).

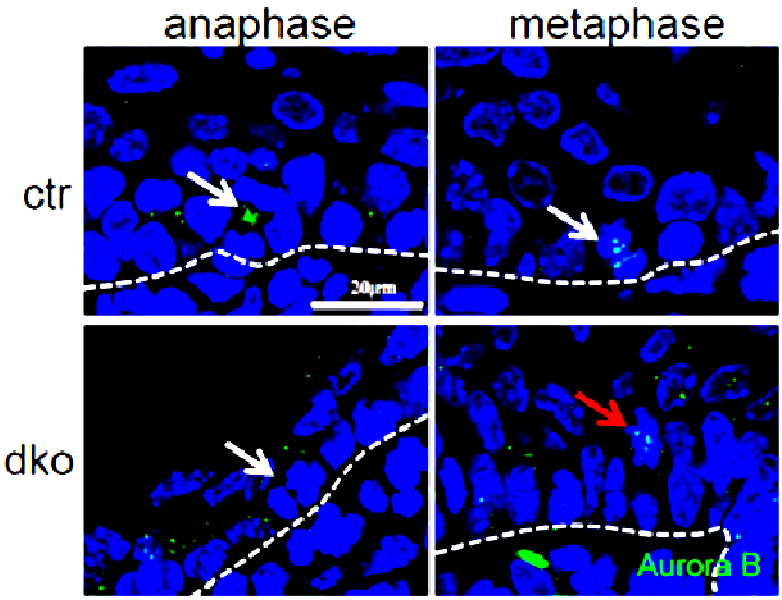

Figure 39: Reduced AuroraB expression in E16.5 dko metaphase and anaphase spindles Immunofluorescence staining of AuroraB (green) on paraffin sections of E16.5 ctr and dko epidermis. DNA was counterstained by DAPI (blue). White lines represent the basement membrane separating epidermis and dermis. White arrows indicate metaphase and anaphase cell divisions in the basal layer and the red arrow indicates a suprabasal metaphase cell. Scale bar is 20 µm. N=3 of each genotype.

2.14 IGF and insulin stimulation is not sufficient to re-activate HeLa cells blocked in G1 and mitosis

To address if IGF-1R signaling is directly involved in mitotic regulation, a method should be established which synchronizes primary keratinocytes in mitosis. Under normal conditions in non-synchronized cells approximately 10% of all cycling cells reside in mitosis, an amount to small for statistical significance. To block or synchronize cells in mitosis we used the microtubule polymerization inhibitor nocodazole which mimics the activation of the mitotic spindle checkpoint. Unfortunately primary keratinocytes are too sensitive for nocodazole and die before synchronization; therefore we performed synchronization experiments with HeLa cells. HeLa cells are derived from epithelial cervical cancer cells which have been well characterized in cell cycle studies. We wanted to analyze whether IGF-1/Insulin stimulation is sufficient to reactivate cells either in S phase, after blocking in G1/S by thymidine, or in mitosis, after blocking spindle formation by a combined treatment of thymidine and nocodazole. Thymidine is a base which in excess is an inhibitor of DNA synthesis and nocodazole, as already mentioned, is a microtubule polymerization inhibitor. To get effective synchronization in mitosis we had to treat the cells with thymidine before adding nocodazole. Untreated HeLa cells showed around 80% of cells residing in G0/G1 phase (Fig.40A) and after thymidine treatment cells revealed an almost 100% synchronization efficiency in the S-phase (Fig.40B). Furthermore, combined treatment of thymidine and nocodazole in HeLa cells led to a 90% synchronization efficiency of cells in mitosis (Fig.40C).

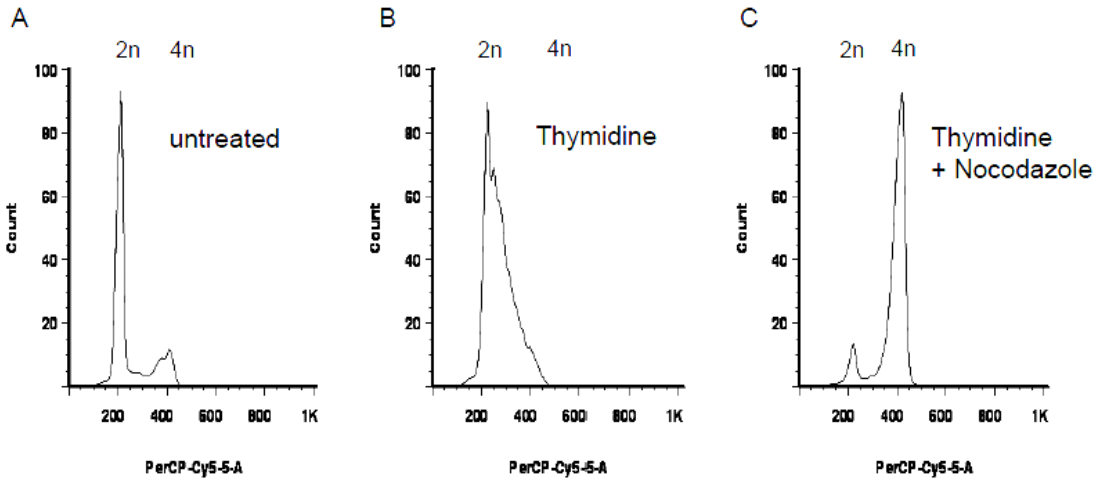

Figure 40: Efficient synchronization experiment in HeLa cells.

Cell cycle analysis of HeLa cells by FACS with propidium iodide (PI). 2n and 4n represent the DNA content measured by PI in fixed cells. X-axis: PerCP-Cy5-5-A represents the PI channel in the FACS machine. (A) HeLa cells untreated. (B): HeLa cells synchronized in S-phase. (C) HeLa cells synchronized in mitosis.

First we were interested whether IGF-1R signaling is sufficient to reactivate synchronized S-phase cells. We chose the indicated time points after washing off thymidine from the cells shown in Fig. 41 to follow the migration of re-activated cells. As expected, after 2h stimulation with FCS we could reactivate synchronized S-phase cells, this was more pronounced after 6h stimulation time (Fig.41A&D). Unlike cells stimulated with either IGF-1/insulin alone (Fig.41C&F) or with starvation medium without FCS these cells cannot re-enter the cell cycle and are still blocked (Fig.41B&E). Contrary to previous findings where IGF-1R signaling drives cells through G1/S phase, the presented results show that in HeLa cells sole IGF-1/Insulin stimulation alone is not sufficient to reactivate synchronized S-phase cells to re-enter the cell cycle.

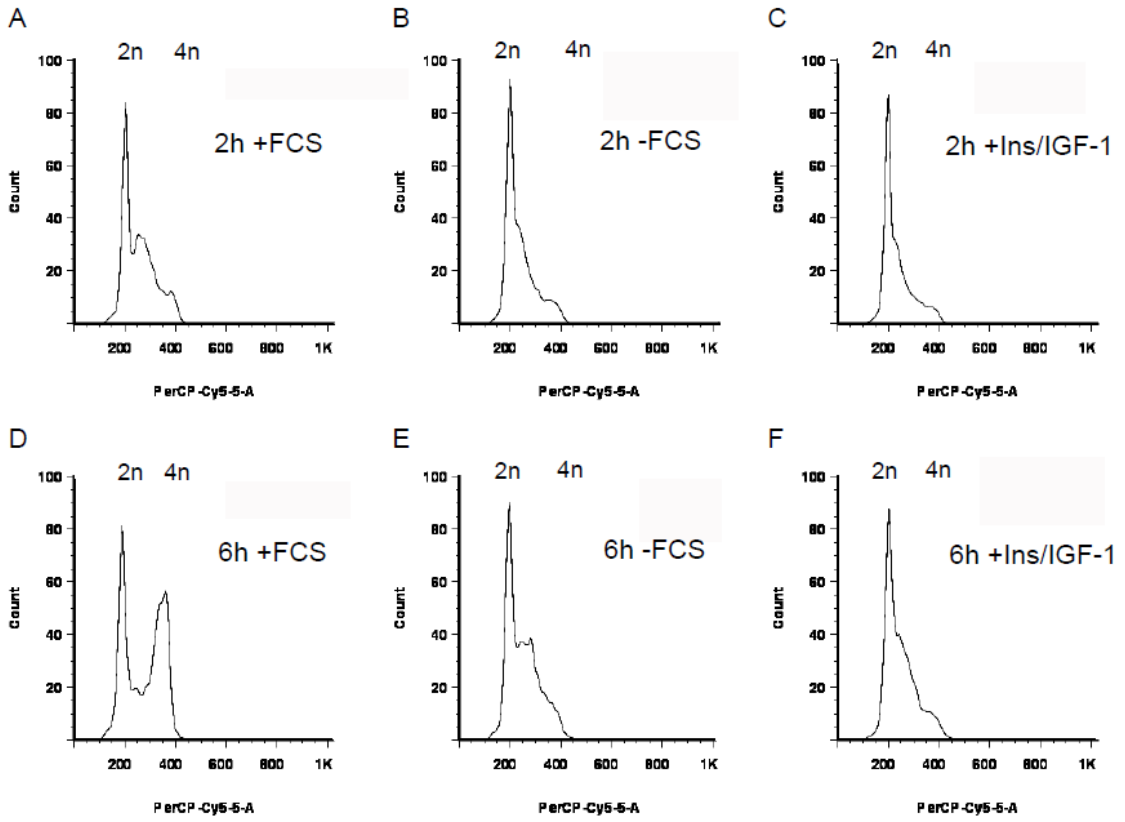

Figure 41: Sole IGF-1/Insulin stimulation is not sufficient to reactivate synchronized S-phase HeLa cells

Cell cycle analysis of HeLa cells by FACS with propidium iodide (PI). 2n and 4n represent the DNA content measured by PI in fixed cells. X-axis: PerCP-Cy5-5-A represents the PI channel in the FACS machine. A-C: HeLa cells treated with thymidine and 2h stimulation with FCS (A, pos ctr), with starvation medium without FCS (B, neg ctr) and Ins/IGF-1 (C). D-F: HeLa cells treated with thymidine and 6h stimulation with FCS (D, pos ctr), with starvation medium without FCS (E, neg ctr) and Ins/IGF-1 (F).

Next, we were interested to know if IGF-1R signaling alone is able to reactivate cells into mitosis after a synthetic spindle block with nocodazole which can be removed from cells by washing-off with PBS. First, we performed western blot of HeLa cells untreated (1), treated with both thymidine and nocodazole (2), treated like (2) but stimulated with starvation medium without FCS (3), treated like (2) but stimulated with insulin/IGF-1 alone (4) and treated like (2) but stimulated with FCS (5). This experiment was performed to investigate whether AKT kinase could be activated after Insulin/IGF-1 or FCS stimulation in nocodazole synchronized HeLa cells (Fig.42). AKT, a well known downstream target of IGF-1R signaling is phosphorylated

after IGF-1R activation in many different tissues. Cells treated with thymidine and nocodazole, blocked mitotic cells and in this experiment the negative control, show below detectable P-AKT activity (Fig.42, lane 2). After stimulation with insulin/IGF-1, the positive control in this experiment, a strong P-AKT activation could be observed (Fig.42, lane 4). This result showed that we are able to both synchronize (inactivate) and reactivate IGF-1R signaling via AKT activation in HeLa cells.
To directly test P-AKT activity in mitosis a shorter stimulation time of FCS is necessary (e.g. 5min-1h), because of the short mitotic stage.

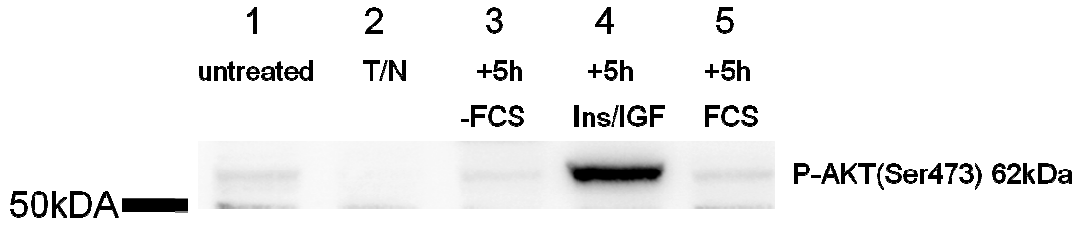

Figure 42: Efficient AKT activation after stimulation with Ins/IGF-1 in synchronized mitotic cells

Western blot analysis for P-AKT (Ser473) on lysates isolated from HeLa cells. Same amount of protein were loaded on SDS gel. Lane1: untreated cells, lane 2: Thymidine/Nocodazole treated cells, lane 3: 5h stimulation without FCS, lane 4: 5h stimulation with Insulin/IGF-1, lane 5: 5h stimulation with FCS.

Cell cycle analysis was also performed on IGF-1/insulin and FCS treated cells, using western blot analysis shown in Figure 42 to see whether IGF-1/Insulin alone is sufficient to reactivate cells after a synthetic spindle block with nocodazole (Fig.43). As expected synchronized cells stimulated with FCS show clear activation of the cell cycle after 5h stimulation (Fig.43A), which was greater after 8h (Fig.43D). Activated cells re-enter the next cell cycle, as seen by the shift from 4n to 2n cells (Fig.43A&D). Cells treated with starvation medium without FCS, originally the negative control which should not reactivate cells, exhibit a slight activation shift from 4n to 2n cells but less than FCS stimulated cells (Fig.43B&E). This background activation probably results from residual growth factors still present in the cell culture system. Cells stimulated with IGF-1/Insulin show the same shift from 4n to 2n cells as the negative control (Fig.43C&F), thus IGF-1R signaling alone is not sufficient to reactivate HeLa cells after synthetic spindle block in mitosis.

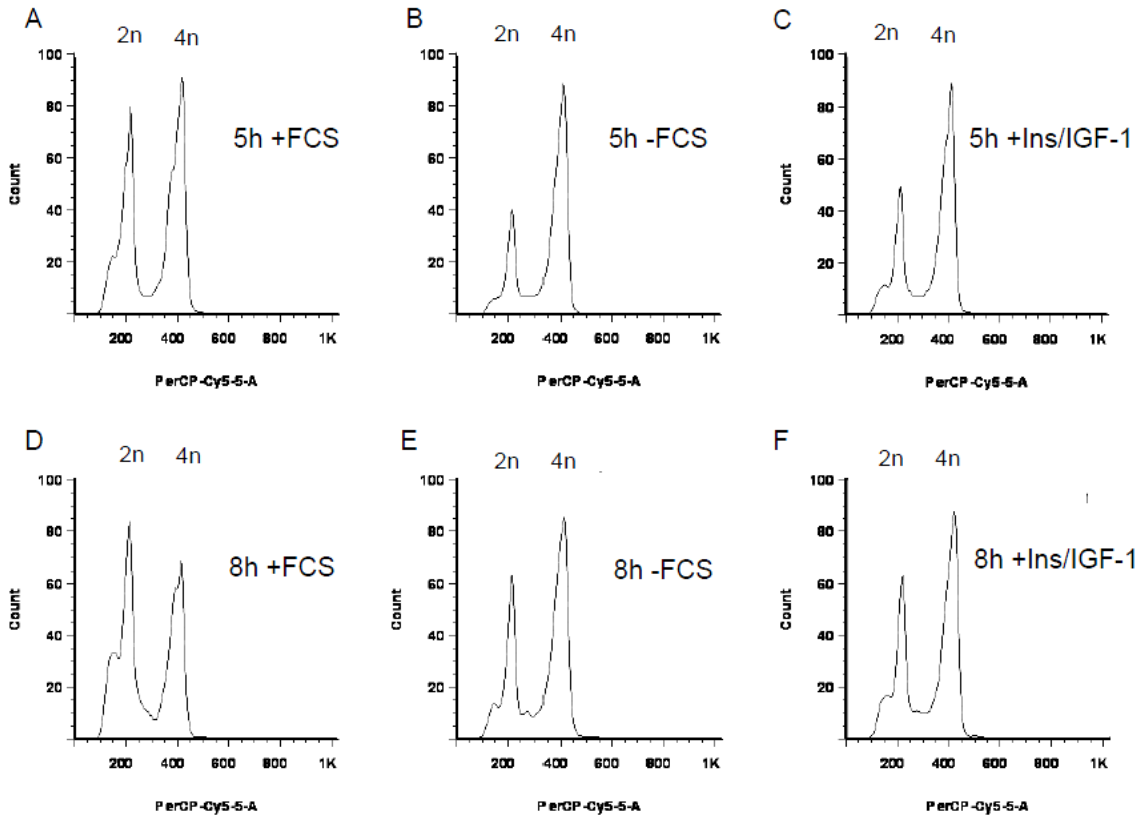

Figure 43: Sole IGF-1/Insulin stimulation is not sufficient to reactivate HeLa cells after the synthetic spindle block with nocodazole

Cell cycle analysis of HeLa cells by FACS with propidium iodide (PI). 2n and 4n represent the DNA content measured by PI in fixed cells. X-axis: PerCP-Cy5-5-A represents the PI channel in the FACS machine. A-C: HeLa cells with combined treatment of thymidine and nocodazole and after 5h stimulation with FCS (A, pos ctr), with starvation medium without FCS (B, neg ctr) and Ins/IGF-1 (C). D-F: HeLa cells with combined treatment of thymidine and nocodazole and after 8h stimulation with FCS (D, pos ctr), with starvation medium without FCS (E, negative ctr) and Ins/IGF-1 (F).

3 Discussion

3.1 The role of IGF-1R signaling in epidermal morphogenesis and proliferative potential

Our study has identified a novel cell autonomous function of both IR and IGF-1R in murine epidermis. In addition, we demonstrate that epidermal IGF-1R, in comparison to IR, is the more important receptor in murine epidermis by regulating epidermal morphogenesis and proliferative potential both *in vivo* during embryonic development and *in vitro* in keratinocytes.

A cell proliferative potential is defined by the number of progeny it can give rise to. Here, loss of proliferative potential was observed in $IR^{-/-}$, $IGF\text{-}1R^{-/-}$ or dko keratinocytes (Fig.13A&B) correlating with the epidermal phenotype. In support of this, it has been shown that IGF-1 restores satellite cell proliferative potential in immobilized aged skeletal muscle (Chakravarthy, Davis et al. 2000) and over expression of IGF-2 expands the number of proliferative units in the epidermis (Bennett, Crew et al. 2003).

Insulin/IGF pathways also regulate epidermal metabolic activity (Wertheimer, Spravchikov et al. 2001) which allows keratinocytes to couple energy capacity directly to proliferative potential of progenitor cells. This may assure self-renewal of the epidermis is adjusted to match its energy level.

However the question remains, how might reduction in proliferative potential result in a hypomorphic epidermis during embryogenesis?

3.1.1 The role of Rac1 in regulating epidermal morphogenesis and proliferative potential

The presented data reveals a key function of insulin and IGF receptor signaling in epidermal morphogenesis, in that the receptors cooperatively determine the number of interfollicular suprabasal layers and proliferative potential of keratinocytes by regulating the small GTPase Rac1.

This protein was previously shown to regulate stem cell maintenance of the epidermis and its appendages (Benitah, Frye et al. 2005; Castilho, Squarize et al. 2007) but the identity of the receptors upstream of Rac1 are not yet determined.

These results show that epidermal Rac1 is activated by insulin and IGF-1 receptors. Furthermore, this activation contributes to epidermal morphogenesis *in vivo* and clonogenic capacity of epidermal keratinocytes *in vitro*.

This is the first *in vivo* evidence that IR and IGF-1R signaling is coupled to the regulation of Rac1. While earlier studies demonstrated that IGF-1R signaling regulates Rac1 activity *in vitro* (Meyer, Kim et al. 2005; Sugimoto, Takuwa et al. 2006) most of these were performed in the context of migration and cytoskeleton alterations and none coupled Rac1 activation to proliferative potential of cells. Importantly, under normal physiological conditions IR and IGF-1R are key activators of Rac1 in the epidermis, since in the absence of both receptors Rac1 activity was reduced by approximately 60-80% (Fig.19).

This strong dependence on IR/IGF-1R activity is surprising in light of the many other receptors and pathways implicated in the regulation of Rac1 (Etienne-Manneville and Hall 2002). These pathways may be more important in directing epidermal Rac1 activity in situations that perturb skin homeostasis, e.g. during wound repair.

By utilizing epidermal specific knockout mice, the most abundant member of the Rac subfamiliy of Rho GTPases in the epidermis, Rac1, has previously been implicated in epidermal stem cell maintenance (Benitah, Frye et al. 2005; Castilho, Squarize et al. 2007), though the data are partially conflicting. Similar to the IGF-1R$^{-/-}$ mice, Benitah and colleagues showed that both the hair follicle compartment and the interfollicular epidermis are affected (Benitah, Frye et al. 2005; Benitah and Watt 2007), whereas two other studies only reported defects in the hair follicle (Chrostek, Wu et al. 2006; Castilho, Squarize et al. 2007). At present the reason for these differential observations is unclear. Epidermal IR/IGF-1R knockout mice do not completely phenocopy the epidermal Rac1$^{-/-}$ mice, in which the IFE is affected. Rac1$^{-/-}$ mice initially showed a phase of hyperproliferation followed by loss of the IFE(Benitah, Frye et al. 2005). Instead, IGF-1R/IR dko mice show an inability to increase the number of suprabasal layers during epidermal morphogenesis, but no further loss of the IFE occurred after birth. This may be due to either residual Rac1 activity (around 20%, Fig.19) in the dko mice or different compensatory signals from the dermis or epidermis which are activated after birth.

Unfortunately, basal expression of RacDA is only able to partially reverse both the *in vitro* loss of proliferative potential and the hypomorphic epidermis *in vivo* seen upon loss of IR and/or IGF-1R. This could be triggered by low expression levels of myc-tagged RacDA, which was detected by western blot (Fig.22A) but not by IF. Additionally, many groups have noted that high levels of RacDA expression are deleterious for cells (Grimsley-Myers, Sipe et al. 2009).

Alternatively, Rac1 is not exclusively responsible for the regulation of proliferative potential during epidermal development. Furthermore, Rac activity and rescue experiments with RacDA were investigated in newborn mice but not in embryos at E16.5 where the phenotype first occurred. Thus, there may be further molecules regulating epidermal development and proliferative potential in collaboration with Rac1 during embryogenesis.

3.1.2 The role of p63 in regulating progenitor cells and proliferative potential during embryonic development

In vivo studies identified the transcription factor p63, a homolog of the p53 tumor suppressor gene, as another key regulator of proliferative potential in epithelial tissue during embryonic development. It has been demonstrated that p63 is strongly expressed in epithelial cells with high clonogenic and proliferative capacity and that stem cells lacking p63 undergo premature proliferative rundown. (Senoo, Pinto et al. 2007). p63 is expressed at E8.5 and expression continues throughout embryonic epidermal development (Kai-Hong, Jun et al. 2007).

In our studies, the reduced expression and focal loss of p63 expression in the basal layer of dko epidermis suggests that IGF-1R/IR signaling regulates individual cells in the interfollicular epidermis (IFE).

Nevertheless, we cannot exclude the possibility that IR/IGF-1R mediate their effect by regulating IFE progenitors, hair follicle stem cells or both. Indeed, the first signs of hypoplasia occur at E16.5 when the primary hair placodes have already formed (E14.5). Epidermal IGF-1R signaling may also have an effect on HF stem cells as surviving IGF-1R$^{-/-}$ mice, but not IR$^{-/-}$ mice, showed hair follicle phenotypes. However, this requires in depth characterization of the mutant hair follicles in adult mice.

On the other hand, several observations suggest that IR/IGF-1R do not affect hair follicle stem cells directly. Firstly key markers for HF stem cells such as CD34 and TCF-4 are not altered in dko newborn mice *in vivo* and secondly, the reduction in label retaining cells in IGF-1R$^{-/-}$ adult mice and the loss of K15 expression in IGF-1R$^{-/-}$ and dko newborn mice were more substantial in the IFE than in hair follicles.

The mechanism by which IR and IGF-1R regulate proliferative potential of epidermal progenitor cells is less clear. IR/IGF-1R most likely do not regulate stem cell maintenance since the IFE phenotype is sustained but does not perturb over time in adult IGF-1R$^{-/-}$ mice (Stachelscheid, Ibrahim et al., 2008). Potentially, epidermal insulin and IGF signaling may regulate the activation of quiescent progenitor cells or set a threshold to the maximum number of cell divisions that progenitor cells can undergo. Alternatively, IR/IGF-1R signaling may act earlier by determining the number of (IFE) progenitor cells during epidermal morphogenesis. A role for IGF-1R/IR in IFE stem cell determination is supported by the observed epidermal loss of keratin 15 (Fig.14) in all three mutant mice and the focal loss of p63 expression even at E16.5. However confirmation of this model requires more detailed comparison to the control interfollicular epidermal progenitor cell population which at present is only poorly defined in the literature (Kaur 2006).
Similar to human embryonic stem cells, which create their own niche by generating IGF-2 secreting fibroblasts and in this manner are able to regulate their own proliferative potential (Bendall, Stewart et al. 2007), we suggest that insulin/IGF-1 signaling might interact with dermal fibroblasts to form the progenitor cell niche in the IFE. At present, it is not known which cells or compartments form the IFE niche.

Together with recent observations in human embryonic stem cells, our results indicate that insulin and IGF-1 receptor signaling via control of Rac1 activity and possibly p63 regulation, may function as crucial set points for the proliferative capacity of stem/progenitor cells in general.
To analyze if p63 is a crucial regulator of proliferative potential for IGF-1R signaling, colony forming assays might be performed to directly compare p63 expression in the different sized colonies of control and IGF-1R$^{-/-}$ keratinocytes. This may reveal if p63 expression is indeed involved in colony formation with high proliferative capacity.

3.2 The role of IGF-1R/IR signaling in the regulation of epidermal proliferation

It has been shown that IR regulates epidermal differentiation (Wertheimer, Spravchikov et al. 2001), whereas IGF-1R signaling contributes to proliferation, apoptosis and differentiation in keratinocytes (Sadagurski, Weingarten et al. 2005). However the studies were performed *in vitro* using keratinocytes isolated from either IR or IGF-1R knockouts and cultured in the absence of feeders. Our data confirm that IGF-1R regulates proliferation during embryonic development but did not reveal any obvious impairment *in vivo* in epidermal differentiation or apoptosis in the absence of IR and IGF-1R. This indicates that although IR and IGF-1R cell autonomously contribute to these processes signals from the dermis can compensate for their loss, thus resulting in normal differentiation and unaltered apoptosis.

Regulation of proliferation is likely to contribute to the mechanism by which epidermal IGF-1R but not IR regulates epidermal development. Strikingly, IGF-1R signaling regulates proliferation only prenatally especially from E16.5 onwards when IFE suprabasal layers increase during epidermal morphogenesis (Lechler and Fuchs 2005). The deletion of either IGF-1R or both IR and IGF-1R receptors (dko) leads to hypomorphic epidermis resulting from a decreased number of suprabasal layers. This is consistent with results supporting that over expression of IGF-1 or IGF-2 results in epidermal hyperproliferation (Ward, Bates et al. 1994; DiGiovanni, Bol et al. 2000). Paradoxically, proliferation defects as shown in IGF-1R$^{-/-}$ and dko mice during embryonic development seem to be rescued in newborn mice, as newborn epidermis exhibits normal proliferation, although still with fewer suprabasal layers. This may be explained by signals coming from the dermis which could compensate for these defects or by delayed mitosis which would be predicted to disturb the accurate stratification during embryonic development.

3.2.1 Mitogenic role of IGF-1R/IR signaling by regulating spindle formation

The interfollicular epidermis (IFE) is continuously regenerated by mitotically active keratinocytes that reside in the inner basal layer and which migrate to the outer cornified layer. During epidermal self–renewal the presence of proliferating basal cells with different proliferative capacity regulates the balance between proliferation and differentiation. Although epidermal IGF-1R/IR signaling coordinates proliferative

potential *in vitro* and epidermal morphogenesis *in vivo* it is still unclear if and how proliferative potential regulates epidermal morphogenesis and how the proliferative potential of cells are connected to epidermal stratification during embryonic development.

An essential process during self-renewing in epidermal development is suggested to be proper mitotic spindle formation and cell division in the basal layer of the epidermis. The increased number of metaphase and decreased anaphase cell divisions in E16.5 IGF-1R$^{-/-}$ epidermis, dko epidermis and IGF-1R$^{-/-}$ keratinocytes suggested a spindle block and provided the first evidence implicating epidermal IGF-1R signaling in the regulation of mitotic progression. Additionally, the increased number of metaphase cells with disturbed spindle formation in IGF-1R$^{-/-}$ keratinocytes strengthens the hypothesis of a spindle block both *in vivo* and *in vitro*.

Our hypothesis that IGF-1R regulates spindle and therefore mitosis progression is in contrast to previous findings in which IGF-1R signaling was involved in G1/S rather than in either G2/M or mitotic stages (Mairet-Coello, Tury et al. 2009) (Stull, Richert et al. 2002). Unfortunately, the limited number of mitotic cells of approximately 10-15% in culture and the inability to perform synchronization experiments with primary keratinocytes prevented the establishment of clear results in IGF-1R$^{-/-}$ keratinocytes. However, *in vitro* synchronization experiments with HeLa cells, immortalized epithelial cell line, excludes that IGF-1R signaling drives cells through G1/S or mitosis, since insulin/IGF-1 stimulation was not sufficient to activate quiescent G1/S or mitotic cells. This was surprising considering previous findings showing a direct involvement of IGF-1 driving cells through G1 and S phase (Ren, Zhong et al. 2009) (Mairet-Coello, Tury et al. 2009) (Stull, Richert et al. 2002). But this is a different system and has to be proofed in primary keratinocytes.

The question remains whether IGF-1R signaling is required in mitosis to either directly or indirectly regulate the spindle checkpoint. So far, nothing is known about the involvement of IGF-1R signaling in spindle checkpoint onset. A single report shows that Insulin receptor is able to interact with an important spindle checkpoint protein (O'Neill, Zhu et al. 1997). It has been published that inhibition and inactivation

of AuroraB results in a spindle checkpoint activation in non-epidermal tissues (Eves, Shapiro et al. 2006), which in line with the altered expression of AuroraB kinase *in vivo* and *in vitro* of IGF-1R$^{-/-}$ and dko epidermis. This strengthens the hypothesis that IGF-1R/IR regulates spindle checkpoint onset in the epidermis, possibly via AuroraB kinase activity. To confirm a direct involvement of IGF-1R/IR in the activation of the spindle checkpoint, *in vitro* and *in vivo* experiments need to be performed investigating an inhibition of e.g. anaphase promoting complex in IGF-1R$^{-/-}$ keratinocytes or in E16.5 epidermis.

We could not exclude yet that expression of other major mitotic regulators are altered after IGF-1R/IR deletion. It has been shown that spindle formation is regulated by specific protein kinases such as PLK1 or AuroraA as well, which are enriched at the centrosomes and involved in centrosomal regulation (Sumara, Gimenez-Abian et al. 2004). Recently, AuroraA has been identified as a downstream target of AKT kinase because inhibition of AKT reduces AuroraA expression resulting in G2/M accumulation, defects in centrosome separation and formation of either monopolar arrays or disorganized spindles (Liu, Shi et al. 2008). These findings resemble the results seen in IGF-1R$^{-/-}$ keratinocytes, which show similar G2/M accumulation and defects in spindle formation as well. So far it remains unclear whether AuroraA and PLK1 expression levels are altered in IGF-1R$^{-/-}$ keratinocytes *in vitro* or in E16.5 dko epidermis *in vivo*. Further experiments have to be performed to follow the expression levels of AuroraA, AuroraB and PLK1 especially in IGF-1R$^{-/-}$ metaphase keratinocytes to determine the causes of the observed phenotype. In general, *in vitro* experiments such as live cell imaging could be done to directly follow cell divisions of individual metaphase cells in IGF-1R-/- and control keratinocytes.

3.4 The role of IGF-1R/IR in asymmetric and symmetric cell division

An essential process in epidermal morphogenesis is the stratification process during embryonic development, occurring from E8.5 onwards to E18.5 (Lechler and Fuchs 2005; Koster and Roop 2007) to form the external, epidermal barrier. Lechler and Fuchs showed that epidermal stratification could be regulated by symmetric vs. asymmetric cell divisions. Interestingly, Lechler and Fuchs could show a functional link between the transcription factor p63 and asymmetric cell division by examining

p63-null embryos. The epidermis of these embryos showed a severe hypomorphic epidermis with an almost complete loss of asymmetric cell division in the epidermal basal layer at E16.5 (Lechler and Fuchs 2005). Furthermore, it has also been reported that AKT kinase is involved in neuronal polarity via asymmetric cell divisions, since AKT activation or inhibition of its degradation led to the formation of multiple axons by disrupting asymmetrical distributions (Yan, Guo et al. 2006).
Overall, the findings in p63-null mice are similar to observations seen in IGF-1R$^{-/-}$ and dko epidermis which at E16.5 also showed hypomorphic epidermis and a loss of basal asymmetric cell divisions. These findings lead to the supposition that IGF-1R/IR signaling could be a potential upstream regulator of p63.

Concomitantly the mitotic regulator AuroraB, which has altered expression in IGF-1R$^{-/-}$ keratinocytes and dko epidermis at E16.5, has been implicated in regulating asymmetric cell division during chromosome alignment in metaphase cells (Chang, Goulding et al. 2006). Therefore, IGF-1R/IR signaling might be a novel upstream regulator of p63 transcription factors and Aurora B kinase coordinating epidermal stratification via asymmetric cell division and regulating proliferative potential.
To examine if p63 networks with AuroraB it would be interesting to investigate if AuroraB expression levels are altered in p63-null mice. Additionally, double staining of p63 and AuroraB in E16.5 control epidermis should be performed to investigate if these molecules co localize especially in metaphase keratinocytes.

In flies and worms, asymmetric cell division produces two daughter cells with different cellular fates, coordinated by unequal distributions of polarity proteins like Numb and Par3/aPKC complex in the two daughter cells. It is still unclear whether polarity proteins are implicated in epidermal asymmetric cell division as well. Lechler and Fuchs could show a connection between asymmetric cell divisions, stratification and an apical organization of LGN and Par3 in mammalian epidermis (Lechler and Fuchs 2005) but no further evidences about polarity proteins regulating asymmetric cell division in mammalian epidermis were documented in the literature. Preliminary results of Numb and aPKC expression in E16.5 by western blot analysis of newborn epidermis did not detect obvious differences between control and dko mice yet (not shown) but a more detailed investigation of, in particular asymmetric cell divisions, in

control and dko E16.5 epidermis is necessary to prove an altered expression of different polarity proteins.

3.5 The role of IGF-1R/IR signaling in regulating epidermal development

At present, we propose a model as shown in Figure 44, summarizing different possibilities, regarding how IGF-1R/IR signaling might coordinate epidermal development and morphogenesis by regulating the increase of suprabasal layers in the epidermis particularly at embryonic stage E16.5.

Firstly we hypothesize that IGF-1R/IR signaling targets p63 thus regulating the proliferative potential of individual basal IFE keratinocytes, possibly in cooperation with Rac1 activation. Additionally, we assume a connection between p63 expression and IGF-1R/IR signaling that regulates stratification and asymmetric cell division since $p63^{-/-}$ null mice, similarly to IGF-1R-/- and dko mice in this study, cannot form asymmetric spindles and lose the ability to generate stratified epithelia during embryogenesis.

Secondly we propose IGF-1R/IR may interact with mitotic regulators like Aurora B, Aurora A or Polo like kinases, mediating precise centrosome organizing and spindle formation in metaphase cells and in part coordinating asymmetric cell division in other tissues. Both the loss of p63 transcription factor in dko epidermis and the altered expression of AuroraB kinase could explain a spindle block due to disturbed spindle formation in E16.5 dko epidermis and IGF-$1R^{-/-}$ keratinocytes. Moreover, the alterations in spindle formation could result in the loss of asymmetric cell division and consequently to fewer suprabasal layers observed in E16.5 IGF-$1R/IR^{-/-}$ epidermis.

So far, it is still an open question whether p63 and AuroraB are downstream mediators of IGF-1R/IR and whether p63 and AuroraB can interact with each other to coordinate the increase of suprabasal layers in epidermal morphogenesis at E16.5.

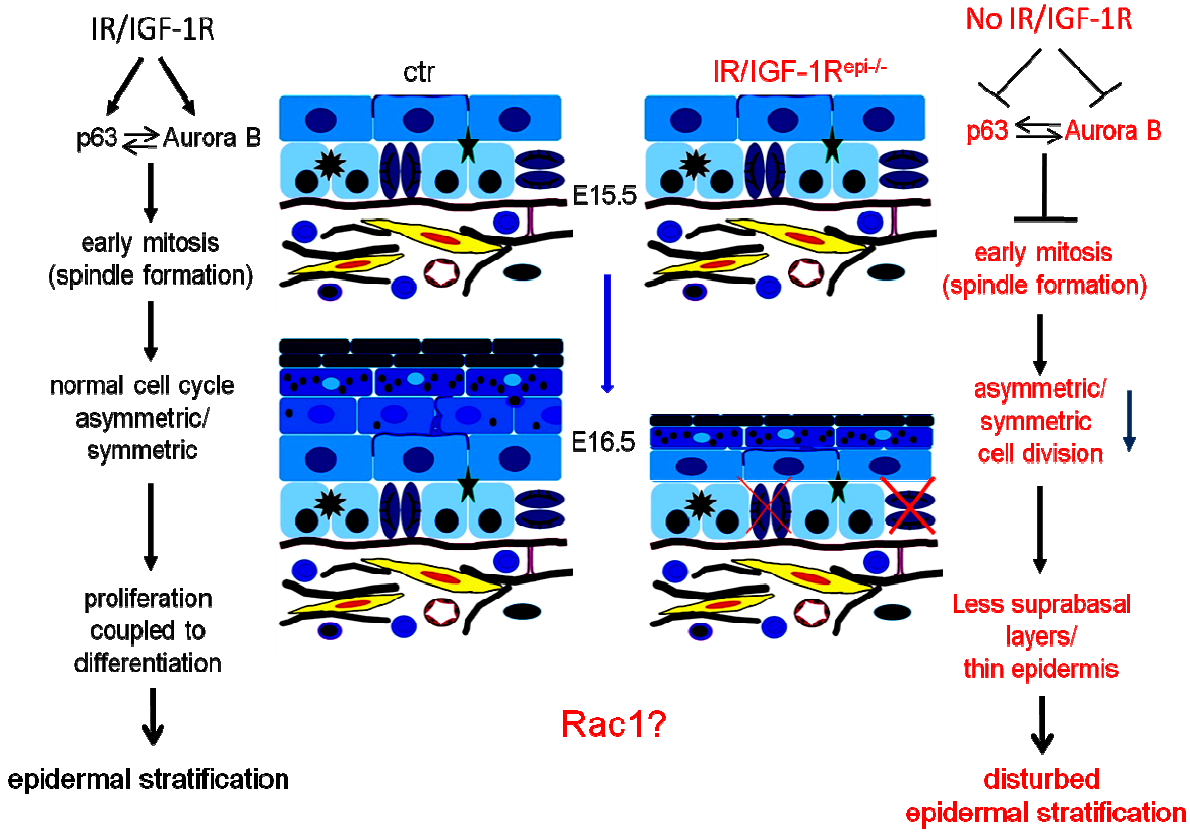

Figure 44: Proposed model how IGF-1R and IR signaling regulate epidermal morphogenesis during embryonic development

P63 and Aurora B could be potential downstream candidates regulating early mitotic progression by forming precise bi-orientated spindles to couple proliferation and differentiation during epidermal stratification. If Rac1 networks with or independently of p63 and AuroraB remains an open question.

From these data we cannot determine whether the small GTPase Rac1 is connected to p63 and Aurora B expression or if Rac1 independently regulates proliferative potential and epidermal morphogenesis within the IGF-1R signaling pathway. This question could be answered by the comparison of IGF-1R$^{-/-}$ cells with and without constitutive RacDA to determine whether RacDA is able to rescue the spindle block *in vitro* or whether the focal loss of p63 can be reversed *in vivo* in IGF-1R$^{-/-}$ basal keratinocytes.

3.6 Network regulation through epidermal IGF-1R/IR signaling

The scheme presented in Figure 45 shows how IGF-1R/IR signaling may network with different molecules by regulating epidermal morphogenesis during embryonic development. The altered expression levels of p63 and Aurora B in epidermal IGF-1R/IR knockout embryos suggest that these molecules may be key targets of IGF-1R and/or IR at E16.5. We have shown that these putative targets are implicated in mitotic spindle formation and/or asymmetric cell division. If and how Aurora B and p63 interact with each other during epidermal IGF-1R signaling could not be shown in this study. Further analysis of IGF-1R$^{-/-}$ mice is required to clarify causes and consequences within the phenotype which would in turn should strengthen our model.

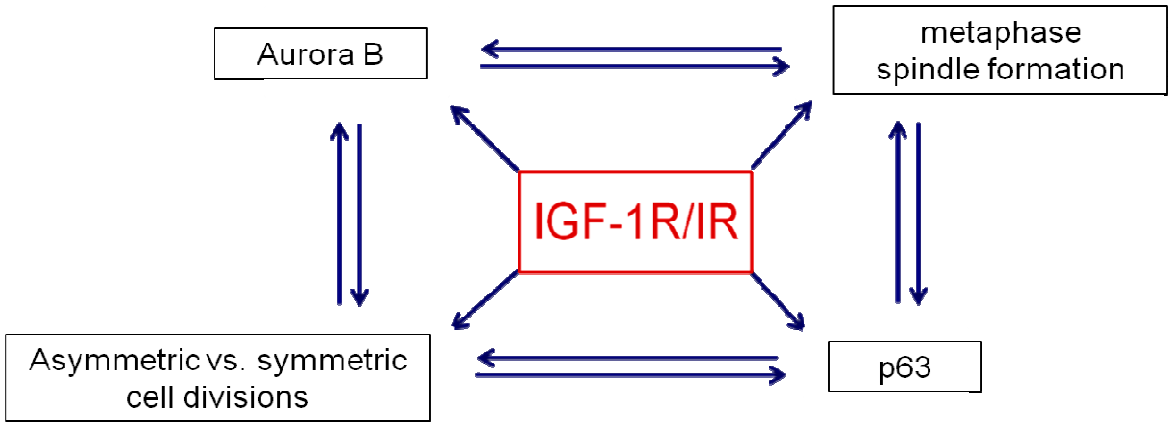

Figure 45: Simplified scheme showing possible networks involved in epidermal IGF-1R/IR signaling during embryonic development.

Aurora B and p63 molecules may be possible downstream targets of the IGF-1R signaling regulating asymmetric versus symmetric cell division and spindle formation in metaphase cell division. It is not known whether these molecules interact with each other to regulate epidermal morphogenesis.

3.7 Open questions & Outlook

In flies it has been shown that mutant of genes such as Aurora and Polo have both spindle orientation defects and increased neuroblast numbers, suggesting that precise spindle alignment is required for normal neuroblast fate. However AuroraA and Polo mutants additionally have cell polarity defects, which complicate the interpretation (Lee, Andersen et al. 2006; Wang, Somers et al. 2006; Wang, Ouyang et al. 2007). In flies and worms cell polarity is regulated through proteins such as Numb, aPKC and Par3 (Petronczki and Knoblich 2001; Cayouette and Raff 2002) though in mammalian epidermis it remains unclear whether these proteins regulate epidermal cell polarity as well. It is still unknown whether polarity is disturbed in IGF-1R$^{-/-}$ and dko E16.5 epidermis and therefore we could not exclude polarity proteins as downstream targets of the IGF-1R/IR signaling, regulating epidermal morphogenesis during embryonic development.

Additionally, we could not show whether the observed spindle block in IGF-1R$^{-/-}$ metaphase keratinocytes and in the epidermis of E16.5 dko mice is indeed specific for IFE progenitor cells. The small but significant number of blocked metaphase cells showing disturbed spindle formation and the focal loss of p63 in basal keratinocytes after IGF-1R/IR deletion would be arguments supporting a role of IGF-1R/IR signaling in spindle formation of individual IFE cell populations, but as mentioned previously, IFE progenitors are not well defined yet.

Moreover if the spindle block in IGF-1R$^{-/-}$ and dko mice at E16.5 is permanent resulting in senescent cells or is the spindle block temporary leading to delayed cell division during embryogenesis. Our results suggest the latter since proliferation defects were only observed at embryonic stages and were no longer present in newborn mice. Further experiments would be required to prove this suggestion.

In addition, we were thus far unable to show whether p53 activation in newborn mice is cause or consequence of the phenotype. Previously, p53 has been implicated in asymmetric cell division and p53 can regulate polarity of cell divisions in mammary stem cells. In addition, it has been shown that loss of p53 favors symmetric divisions of cancer stem cells contributing to tumor growth (Cicalese, Bonizzi et al. 2009). Furthermore, p53 is an important cell cycle regulator, coordinating precise cell division through the cell cycle and is also known as “the

guardian of the genome". It would be interesting to investigate p53 expression levels in either primary keratinocytes or E16.5 epidermis, especially in metaphase cell divisions, to see if p53 may also be involved in epidermal spindle positioning and orientation.

Moreover, it has been shown that m-Tor and its targets regulate cell cycle events like cell growth and proliferation in other tissues (Fingar, Richardson et al. 2004). The m-Tor signaling pathway could not be identified as a downstream target of epidermal IGF-1R/IR because preliminary western blot experiments did not show clear results. It would be interesting to examine m-Tor signaling in E16.5 control and dko mice as m-Tor may be involved in epidermal cell cycle regulation and consequently could directly affect the cell cycle in dko epidermis at E16.5.

In summary, we show that IGF-1R/IR signaling has an important cell autonomous function in epidermal development during embryogenesis. In the future, studies should be performed to answer whether IGF-1R/IR signaling has cell autonomous function in adult epidermis. Previously, we could show that hyperproliferative pulses of TPA lead to a dampening response in adult IGF-1R$^{-/-}$ epidermis (Garinis, Uittenboogaard et al. 2009). Thus, the result suggests that epidermal IGF-1R/IR signaling might be a key regulator during hyperproliferative responses and therefore a potential regulator involved in the development of skin deseases and cancer. Additionally, in order to address the main function of IGF-1R signaling at different developmental stages in adult mice, we will employ an inducible system in mice expressing the tamoxifen-inducible K14Cre promotor as well as homozygous IGF-1R floxed alleles. This provides a deletion of IGF-1R at specific time points in adult epidermis.

4 Materials and Methods

4.1 Mice

The generation and genotyping of the insulin receptor floxed mice and the K14-Cre mice have been described (Bruning, Michael et al. 1998; Hafner, Wenk et al. 2004). To generate IGF-1R floxed mice, a targeting vector was constructed encompassing 4.5 kb of intron 2 of the murine IGF-1R gene, a loxP site, an FRT-flanked neomycin resistance gene, 600 bp fragment containing exon 3, a second loxP site, and 1.3 kb of intron 3. Cre-mediated recombination and subsequent excision of exon 3 of the IGF-1R results in a frame shift after 213 codons, with an appended sequence of 27 amino acids followed by a stop codon in exon 4. Gene targeting was performed in Bruce4 ES cells (Kontgen and Stewart, 1993). For transfection, $1x10^7$ ES cells were transfected with 40 mg DNA as previously described (Brüning et al, 1998). Approximately 9 days after transfection, G418/Ganciclovir double-resistant colonies were picked and expanded on 96-well tissue culture dishes. Genomic DNA was extracted from each clone and analysed by Southern blot and PCR analysis. Recovery, microinjection and transfer of 3.5 day p.c. embryos were performed according to standard procedures. Chimeric animals (80-90% chimerism based on coat color) were bred with FlpE deleter on a C57BL/6 background. Genotyping was performed by PCR on DNA extracted from tail biopsies using customized primers: 5'-TCC CTC AGG CTT CAT CCG CAA -3' (sense) and 5'- CTT CAG CTT TGC AGG TGC ACG -3' (antisense). IGF-1R floxed mice and the K14-Cre mice are in Bl6, the IR floxed mice and the $dko^{-/-}$ mice are Bl6 mixed Bl6 in the 4th to 5th generation. The generation of K14-RacL61myc mice (K14-RacDA) will be described elsewhere. These mice have no obvious phenotype. To perform *in vivo* Rac rescue experiments the K14-Rac were first crosssed to K14-Cre mice to generate K14-Cre;K14-RacDa mice, which were crossed to IGF-1R fl/fl mice to obtain male K14-Cre/+ ;IGF-1R fl/+;K14-RacDA mice. These mice were subsequently crossed with IGF-1R fl/fl females to generate; $IGF\text{-}1R^{-/-}$; K14-RacDA as well as $IGF\text{-}1R^{-/-}$ mice. Analysis was done on 5 mice of each genotype.

4.2 Keratinocyte cell culture

The experiments described were performed with primary keratinocytes isolated from K14Cre/+; IGF-1Rfl/fl (IGF-1R-/-) and IGF-1Rfl/fl (ctr) mice.
All experiments were done under sterile conditions.

4.2.1 Isolation of primary keratinocytes

Newborn mice were sacrificed by decapitation, the bodies were incubated for 30 min on ice. Subsequent sterilizing washes (iodine solution, sterile PBS, 70% EtOH, each for 1 min), the skin was carefully removed from the torso and the tails was prepared for genotyping. The skin was floated dermal side down in a 0,6 cm cell culture dish filled with 1 ml of cold 0.25% trypsin solution and incubated at 4℃ for 15 to 24 hrs. Each skin was transferred to a dry, sterile culture plate and spread out with the dermis facing down. The epidermis was seperated from the dermis by using foreceps and the epidermis was cut in little pieces with 2 scalpel blades, transferred to 2 ml eppendorf tube containing 1,5 ml prewarmed growth medium. Well suspensions were shaked for 30 min at 32℃ and subsequently plated o n collagen-coated 6-well plates. The primary mouse keratinocytes were cultured at 32℃, 5% CO_2 before use or further passaging.

4.2.2 Culture of primary keratinocytes

Primary keratinocytes were cultured on collagen type-1 (Biochrom, Berlin, Germany) coated dishes in FAD medium containing 50µM Ca^{2+} in the presence of mitomycin C-treated (4µg/ml) 3T3 fibroblasts as feeders. Fibroblasts were cultivated on 10 cm plates and maintained in an incubator with a humified atmosphere at 37℃ and 5% CO_2.

4.2.3 Colony forming assays of primary keratinocytes

$4x10^4$ primary keratinocytes (passage 0-3) were plated in triplicates in a 6-well plate and cultured for approximately 3 weeks in the presence of fibroblasts. Fibroblasts were changed twice a week. Only keratinocytes isolated from littermates were compared. Cells were fixed with 1% PFA for 15min and susequently stained for minimum 1h with 0.05% crystal violet in PBS. Digital images were obtained for

analysis of colony size and number using ImageJ. For each knockout line experiment were repeated at least three times with independently isolated keratinocytes.

4.2.4 Transduction of primary keratinocytes

For RacDA rescue experiments IGF-1R$^{-/-}$ or dko cells were transduced with lentivirus containing either mycRacL61 (RacDA) or GFP by incubating cells o/n with supernatant containing particles. Cells were then plated for colony forming assays. Lentiviral particles were obtained by transient transfection of 293 FT cells with CMV-mycRacL61 or GFP lentiviral constructs and the virapower packaging mix (Invitrogen) using lipofectamine 2000. Cells were grown in FAD medium and supernatant containing viral particles were collected each day for three days after which they were pooled. Three independent transduction experiments in combination with colony forming assays were performed.

4.2.5 Cell cycle synchronization with thymidine and nocodazole

1x10^6 cells/ well were seeded into a 6cm plate. After 4 h, cells were treated with 2mM thymidine (Sigma) Thymidine stock solution: 10mM in DMEM (sterile filtered). After 23h, cells were washed three times with PBS. Then cells were treated with 5µg/ml nocodazole (Sigma; stock solution: 1mg/ml in DMSO) for 5h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% streptomycin, 1% penicillin and after three time washing with PBS, the medium was exchanged by DMEM without supplements (starvation medium) for another 12-15h. After incubation, cells were washed three times (3x10min) with PBS. After nocodazole treatment and washing process, cell were reactivated with either normal medium, starvation medium or Insulin/IGF(10nm) at different time points. Finally cells were harvested and fixed with 70% ETOH/PBS for FACS analysis or lysed in RIPA buffer for western blot analysis.

4.2.6 Cell cycle analysis by flow cytometry (FACS)

Cells were harvested by treatment with trypsin/EDTA, washed in 5ml PBS, and resuspended in 3ml PBS. After adding 7 ml 100% ethanol cells were fixed and incubated at 4℃ for minimum 2 h and then centrifug ed for 7 min at 1000rpm. The pellet was 2x washed with PBS, filtered with a 70µm cell strainer and resuspended in

400 µl PBS containing 0.1% Triton X-100, RNAse A (10 mg/ml) and propidium iodide (2 mg/ml, Sigma #P4170). Stock of 5ml: 5µl Triton-X (5µl), 100µl RNAse A and 50µl Propidium iodide in PBS. Than cells were stained in the dark at 37℃ for 15min and analyzed by FACS (FACScalibur, BD biosciences).

Reagents and media for keratinocyte cell culture

1) DMEM (FAD)-medium (Growth medium for keratinocytes)

- 10% FCS, treated with Chelex
- 0.4 µg/ml hydrocortisone
- 5 µg/ml insulin
- 10 ng/ml EGF
- 10^{-10} M cholera toxin
- 100 U/ml penicillin and 100 µg/ml streptomycin
- 2 mM L-Glutamin

2) Dulbecco`s phosphate buffered saline without calcium and magnesium

3) Trypsin: 1 x Trypsin/EDTA 0.05%

4.3 Protein biochemical methods

4.3.1 Isolation of the epidermis of newborn mice

Epidermis was separated from the dermis by floating skin biopsies, epidermis side up, in a 0.5 M ammoinium thiocyanate (NH_4SCN) in phosphate buffer, pH 6,8 (0,1M Na_2HPO_4, 0,1M KH_2PO_4) for 20 min on ice. Epidermis was either snap frozen in liquid nitrogen or immediately processed for RNA isolation or processed in protein lysates.

4.3.2 Protein extraction from epidermal splits and keratinocytes

Cultured keratinocytes or skin tissue were lysed in 1% Ripa lysis buffer supplemented with mammalian protease inhibitor cocktail (Sigma-Aldrich) and 2 mM PMSF (Fluka) as well as phosphatase inhibitors to prevent protein degradation by endogenous proteases or phosphatases during lysis.

Epidermal lysates were generated by homogenizing the tissue by a mixer mill (Qiagen) for 3 min at 30Hz. Cells were washed twice with PBS before adding the

lysis buffer and were harvested with a cell scraper and transferred into a reaction tube. Afterwards lysates were rotated for 1h at 4℃ and were centrifuged for 10min at 13000rpm and 4℃ and supernatants were transferred into a new reaction tube.

1% Ripa buffer
150mM NaCl
50mM Tris pH7.4
4mM EDTA
1% NP40
0.5% DOC (Natriumdeoxycholsäure)
ddH_2O

4.3.3 Protein determination with the modified Lowry method

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. It is named for the biochemist Oliver H. Lowry who developed the technique in the 1940s. The principle of this method is that under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

4.3.4 SDS-polyacrylamid-gelelectrophoresis (SDS-PAGE)

Denaturized proteins were separated according to their molecular mass via SDS-polyacrylamid-gelelectrophoresis. Samples were dissolved in SDS-sample buffer and incubated for 5 min at 95℃. 50 µg protein concentr ations were loaded on the gels, which were separated at 40mA per gel for an appropriate time in 1x SDS running buffer. Depending on the molecular mass of the proteins of interest, gels containing 6-12% acrylamid were used. In some cases, commercially available gradient gels (4-12% gradient Bis-Tris Gels, Invitrogen) were applied.

4.3.5 Western Blot Analysis

Epidermis was separated from the dermis and grinded in a mixer mill 300 (Qiagen) in lysis buffer without detergent after which they were lysed in lysis buffer containing 1% NP-40, 0.5% deoxycholate, 0.2% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.4, and a cocktail of protease inhibitors (Sigma). Equal amounts of total protein (30-50 ug) were separated by SDS-PAGE using NuPage 4-12% gradient Bis-Tris Gel (Invitrogen), transferred to nitrocellulose (G&E, Freiburg, Germany) and probed with the appropriate antibodies. If the protein size of interest was more than 10 kD different from actin, actin was probed on the same filter. Otherwise, a gel was run simultaneously using the same protein amounts and probed for actin, to assure that similar amounts were indeed present.

4.3.6 Rac activation assay

Skin biopsies were taken from mice, suspended in lysis buffer containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, protease inhibitor cocktail (Sigma) and 4 µg biotin-CRIB-Pak peptide. After grinding of the tissue in a mixer mill 300 (Qiagen), 1 %NP-40 was added and incubated for 1 hour at 4ºC. After centrifugation for 15' at 4ºC and removing total lysate aliqots, supernatants were rotated for 1 hour at 4ºC, streptavidin-agarose beads added and rotated for another hour. Beads were washed three times with lysis buffer and bead samples and equal amounts of total lysate were analyzed by western blot.

4.3.7 Gene expression analysis

Gene expression was analyzed using quantitative RT-PCR. RNA was extracted from epidermis using RNeasy Minikit (Qiagen) according to the manufacturers instructions. Epidermal RNA was reversely transcribed with SuperScript Reverse Transcriptase (Invitrogen) and amplified using TaqMan Universal PCR Master Mix. Quantitative PCR was performed on an ABI-PRISM 7700 Sequence Detector (Applied Biosystems). Assays were linear over 4 orders of magnitude. Calculations were performed by a comparative cycle threshold (Ct) method: the starting copy number of test samples was determined in comparison with the known copy number of the calibrator sample (ddCt). The relative gene copy number was calculated as 2–ddCt. Probes for target genes were from TaqMan Assay-on-Demand kits (Applied

Biosystems). Samples were adjusted for total RNA content by HPrT or TATA binding protein.

4.4 Immunohistochemistry

Fluorescent stainings were analyzed with the Olympus IX71 microscope in combination with the DeltaVision system (Chromaphore) and with the confocal microscope Zeiss Meta 510. The pictures were recorded with the digital camera cool snap^{TM} HQ^{2} (photometrics) and stored with the software Softworx 3.6.1 (Applied Precision) and pictures were processed with Adobe Photoshop and Illustrator. Statistical analyses were done using Microsoft Exel and GraphPad Prism 5.

4.4.1 Paraffin embedded skin sections

Skin sections were removed from the dorsal section of the mouse back and fixed in 5% formalin saline for 12h at 4℃. After dehydratio n of the tissue the skin sections were paraffin embedded and stored at room temperature until required. Paraffin embedded skin sections were cut into 6µm thick slices and dried on glass coverslips. The sections were deparaffinized and were washed twice in PBS. Afterwards the tissue was unmasked using the antigen retrieval (EMS).

Demasking buffer
0,1M Citric acid
0,1M Na-citrat
Aqua dest

Subsequently, the sections were blocked with 10% serum derived from the species from which the secondary antibody was taken. The sections were incubated for 1h at room temperature in a humified chamber. After washing the samples with PBS the slides were incubated in a humified chamber 1h or overnight with the primary antibody diluted in blocking solution. After washing step in PBS, the slides were incubated with the secondary antibody coupled to Alexa 488 or Alexa 594 diluted in blocking solution. Nuclei were counterstained using propidium iodide or Dapi (Sigma Aldrich). After a final washing, the slides were mounted and stored at 4℃ in the dark.

4.4.2 Frozen skin sections/keratinocytes

Skin was removed from the dorsal region of mice and immediately embedded in OCT mounting media and placed on dry ice for cryosectioning. The cryosections (6µm) were fixed in ice-cold methanol or aceton for 10 min at room temperature.

Keratinocytes were cultured on collagen coated cover slips prior to 10 min fixation with methanol or 4%PFA.

Tissue or cells were blocked in PBS containing 1% BSA and in case of PFA fixation 0.02% Tween 20 for 1h in a humified chamber. After washing with blocking solution the cover slips were incubated with the primary antibody diluted in blocking solution 1h or overnight at 4℃ in a humified chamber. After washing in PBS the slides/cover slips were incubated with the secondary antibody coupled to Alexa 488 or Alexa 594 for 1h diluted in blocking solution. Nuclei were counterstained using propidium iodide or Dapi (Sigma Aldrich). After a final washing, the slides were mounted and stored at 4℃ in the dark.

4.4.3 Preparation of whole mount tail skin

Whole mounts of mouse tail epidermis were prepared as previously described (Braun and Watt 2004). Briefly, whole skin was removed from the tail of mice and incubated for 3 hours at 37^{o}C in 5mM EDTA/PBS. The epidermis was then separated from the dermis and fixed in 4% formal saline for 2 hours at room temperature and stained.

Photos were taken with a NIKON Eclipse E800 microscope equipped with a NIKON DMZ1200 camera or a LEICA confocal microscope. K15 staining was quantified by counting all K15 positive hair follicles and interfollicular epidermal cells in a 500 µm^2 area. Likewise, BrdU positive cells in the hairfollicles and interfollicular epidermis in a 500 µm^2 area were counted in sections or whole mounts.

4.4.4 BrdU labeling of label retaining cells

Short-term BrdU (5-bromo-2'-deoxyuridine) labeling experiments were performed to visualize cells transiting in the epidermis. Postnatal mice were injected intraperitoneally with100 mg/kg bodyweight BrdU and sacrificed following a 24h chase period. To compare populations of quiescent label-retaining cells (LRC) te-days-old mice were injected with 50 mg/kg bodyweight BrdU every 24 h for 3 injections. Following a 15, 40 or 70 day chase period the mice were sacrificed and

dorsal skin (day15) and tail skin whole mounts (day 40 and day 70) were isolated for immunostaining and analysis.

4.5 Antibodies

4.5.1 Primary antibodies

Antigene	Source	Working dilution	Catalog number	company
Aurora A	mouse	IF: 1:500 (MetOH)	Ab1287	Abcam
Aurora B	rabbit	IF: 1:150 WB: 1:2000	Ab2252	Abcam
P-Aurora A,B,C	rabbit	WB: 1:1000	2914S	Cell signaling
c-Myc	mouse	WB: 12,5µl/ml	OP10	9E10, Calbiochem
14-3-3σ	goat	WB: 1:750	Sc7681	N14, Santa Cruz
P53	mouse	WB: 1:1000	2524	Cell signaling
P38	rabbit	IF: 1:100 WB: 1:1000	9212	Cell signaling
p-p38	mouse	WB: 1:2000	9216	Cell signaling
Rac1	mouse	WB: 1:1000	R2650	23A8, Sigma
Akt	rabbit	WB: 1:1000	2938S	Cell signaling
P-Akt (Ser473)	rabbit	WB: 1:1000	4060S	Cell signaling
P-Akt (Thr308)	rabbit	WB: 1:1000	2965S	Cell signaling
K15	chicken	IF: 1:750 WB: 1:2000	Lot: 14920101	Covance
BrdU	mouse	1: 20	347580	BD
P63	mouse	WB: 1:2000	M7247	BD
HES1	rabbit	WB: 1:2500		from N. Brown, Cincinnati, Ohio
Ki67	mouse	IF: 1:1000	TEC-3	TEC-3, DAKO
actin	mouse	WB 1:10000		C4, Sigma Aldrich

α-tubulin	mouse	IF: 1:20	CP06	Calbiochem
γ-tubulin	rabbit	IF: 1:100	11321	abcam

4.5.2 Secondary antibodies

Antigene	**Source**	**Working dilution**	**Catalog number**	**company**
Alexafluor 488 anti-mouse	goat	IF 1:500		Molecular Probes
Alexafluor 488 anti-rabbit	goat	IF 1:500	A21206	Molecular Probes
Alexafluor 594 anti-mouse	goat	IF 1:500		Molecular Probes
IgG-HRP anti-rabbit	goat	WB 1:5000		BioRad
IgG-HRP anti-mouse	goat	WB 1:5000		BioRad
IgG-HRP anti-goat	goat	WB 1:5000	Sc2020	Santa Cruz
IgG-mouse anti rabbit	rabbit	IF:1:10	0931	Dako

Table1: Antibodies, used for specific detection in western blot and immunohistochemistry. Their source, working dilution, the specific clone and producing company are shown. WB: western blot, IF: immunofluorescence

5 Abbreviations

Akt	proteinkinase B
BSA	bovine serum albumin
Bp	base pairs
BrdU	bromdesoxyuridin
℃	degree Celsius
cDNA	complementary DNA
Cre	site specific recombinase from phage P1(causes recombination)
DAPI	4`6-Diamidino-2-phenylindol
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
E	embryonic day
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig	figure
floxed/ lox	lox P flanked
Foxo1	forkhead-O transcription factor 1
FSC	forward scatter
GFP	green fluorescent protein
GTP	Guanosintriphosphat
g	gram
h	hours
H&E	hematoxylin/eosin
HeLa	cell line derived from cervical cancer cells taken from Henrietta Lacks
HF	hair follicle
HRP	horse radish peroxidase

Hz	hertz
IF	immunofluorescent
IFE	interfollicular epidermis
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1receptor
IR	insulin receptor
K	keratin
Ki67	Kiel 67 (university of kiel)
kD	kilo Dalton
KO	knockout
LRC	label retaining cells
MAPK	mitogen-activated protein kinase
mg	milligram
min	minute
µl	micro liter
ml	milliliter
µm	micro molar
mM	millimolar
mRNA	messenger RNA
n	nano
NGS	normal goat serum
P	postnatal day
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	phosphoinositide-dependent protein kinase 1
PI3K	phosphatidylinositol 3 kinase
PIP_2	phosphatidylinositol (4,5) biphosphate
PIP_3	phosphatidylinositol (3,4,5) triphosphate
PFA	paraformaldehyde
PMSF	phenylmethylsulphonylfluorid
Rac1	Ras-related C3 botulinum toxin substrate 1
RNA	ribonucleic acid

RNAi	RNA interference
RNase	ribonuclease
RT	real time
rpm	rounds per minute
SDS	sodium dodecyl sulphate
sec	seconds
SSC	side scatter
TBS	tris buffered saline
TE	Tris-EDTA buffered
Tween	polyoxethylene-sorbitan-monolaureate
U	unit
vs.	versus
v/v	volume/volume
w/v	weight per volume
WB	western blot

6 Companies

Amersham Biosciences AB, Uppsala, Sweden

Abcam, Cambridge, UK

BioRad Laboratories GmbH, München, Germany

Carl-Roth GmbH & Co, Karlsruhe, Germany

New England Biolabs, Beverly, USA

Fluka (BioChemika), Buchs, Switzerland

Invitrogen GmbH, Karlsruhe, Germany

Millipore, Bedford, USA

PEQLab Biotechnology GmbH, Erlangen, Germany

Pierce, Rockford, USA

Promega GmbH, Mannheim, Germany

Qiagen, Hilden, Germany

Santa Cruz Biotechnology, Santa Cruz, USA

Science Products GmbH, Hofheim, Germany

Serva Electrophoresis GmbH, Heidelberg, Germany

Sigma-Aldrich Chemie GmbH, München, Germany

Stratagene, Amsterdam, Netherlands

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9 Erklärung

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