

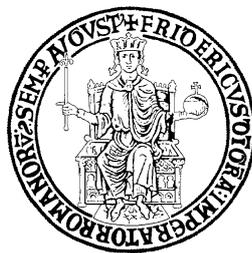
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**Doctorate School in Molecular Medicine**

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**EVIDENCE FOR ALTERATED WNT AND  
NOTCH SIGNALING IN A H-PRUNE  
TRANSGENIC MOUSE MODEL**

**DE VITA GENNARO**



**Napoli 2011**

*“Truth is into the shades”*

**Henry Charles Bukowski**

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## Abstract

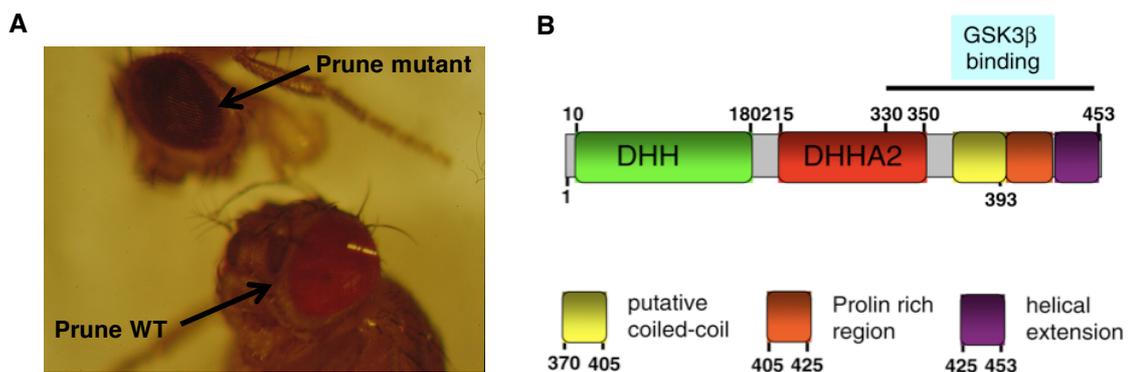
H-Prune protein stimulates cellular motility and metastasis process of breast cancer cells. The role of h-Prune in increased cell motility can be explained in part to h-Prune interaction with GSK-3 $\beta$  with which cooperatively works to regulate the disassembly of focal adhesions and promote cell migration. In the present study we use a transgenic mouse model to investigate the role of h-Prune in the epidermis. We uncover defects in stratification, differentiation and barrier formation which can be attributed to a failure in basal keratinocytes proliferation and migration of ML-h-Prune transgenic mice. We highlight a role of h-Prune in sustaining Wnt/ $\beta$ -catenin pathway thus investigating the molecular mechanism underlying the observed phenotype. We notice that h-Prune overexpression lead to higher rate of GSK3- $\beta$  phosphorylation resulting in its inactivation. To follow the Wnt pathway variation mediated by Prune *in vivo*, we generate a double transgenic mice model pVegfr2-luc/ML-h-Prune that shows an higher signal of bioluminescence driven by the pVegfr2 promoter, in agreement of the maximum peak of h-Prune expression. Thus these analyses *in vivo* show that h-Prune is able to enhance VEGF- $\alpha$ , through its sustaining activation of the WNT signaling in the mouse skin. Because of the knowledge of Notch signaling involved into an altered differentiation pattern of keratinocytes, we observed in the ML-h-Prune transgenic skin an unbalanced differentiation phenotype, thus we decide to analyze the level of the Notch pathway activation in transgenic mice skin. We show here a substantial reduction of downstream effectors of these signaling in the h-Prune transgenic animals . The phenotype of our transgenic model is very close to an inflammatory phenotype, with unbalancing levels of both WNT and Notch pathways, thus postulating a correlation of h-Prune overexpression and consequent inflammation into the mice skin. For these reasons we analyzed h-Prune together with  $\beta$ -catenin nuclearization in serial sections of human psoriatic skin, asserting an involvement of h-Prune in these pathology, a disease which retains both proinflammatory insult and higher WNT-activated proliferation phenotype. This model could be the base for future studies that will assess properties and functions of h-Prune in human disorders which are related to hyperproliferation and inflammation of the skin. This is an attempt to find molecular targets for future therapeutic applications in the treatment of inflammatory skin pathologies.

# **1.BACKGROUND**

## 1.1.H-Prune

H-Prune is the human homologue of the *Drosophila* Prune gene was originally characterized based on *Drosophila* mutant phenotypes. Prune (or DRES17) was identified as a gene that is involved in eye development in a *Drosophila* model, starting from a subset of human ESTs homologous to *Drosophila* mutant genes called *Drosophila*-related expressed sequences (DRES) [Banfi, et al. 1996]. The *Drosophila* Prune mutant showed a brownish-purple eye colour due to a reduction in drospteridines, in contrast to the bright red eye of the wild type fly [Timmons and Shearn 1996]. The metabolic defects in the Prune fly do not affect viability and fertility; however, the homozygous or hemizygous Prune mutation is lethal in the presence of a single copy mutation of the abnormal wing disc gene (*awd*; also known as killer-of-Prune).

The product of h-Prune gene is a protein that belongs to the DHH protein superfamily, which includes several phosphodiesterases (PDEs), such as RecJ, a bacterial nuclease, and different yeast and bacteria pyrophosphatases [Aravind and Koonin 1998]. The DHH superfamily can be divided into two main groups on the basis of a C-terminal motif that is very well conserved within each group, but not across the groups. All the members of this superfamily possess four other motifs that contain highly conserved charged residues which are predicted to be responsible for ion binding and for catalysing the PDE reaction. The most characteristic motif is the third, with the signature DHH (Asp-His-His), after which this superfamily was named. At N-terminus of the h-Prune sequence (residues 10–180) is located the DHH domain, along with an adjacent, less conserved, DHHA2 domain [D'Angelo, et al. 2004] that provides substrate-binding residues and therefore influences specificity. The DHHA2 domain is therefore important in studies of possible substrates of h-Prune. This domain is followed by a C-terminal cortexillin-homology region with putative coiled-coil and proline-rich regions.



**Fig 1. *Drosophila* Prune mutant and Molecular domains of h-Prune.** **A.** *Drosophila* Prune mutant showed a brownish-purple eye colour due to a reduction in drospteridines, in contrast to the bright red eye of the wildtype fly [Timmons and Shearn 1996]. **B.** The N-terminus of the h-Prune sequence contains the DHH (residues 10–180) and DHHA2 (residues 215–350) domains involved in its enzymatic function. These domains are followed by a cortexillin-homology domain (CHD, 370–453 residues) containing putative coiled-coil and proline-rich regions. The C-terminal region of h-Prune is responsible for the h-Prune interaction with GSK-3 $\beta$  (330–453 residues).

The human *PRUNE* gene is ubiquitously expressed in adult tissues, while its expression during mouse development is particularly high in the central nervous system (CNS), specifically in the cranial and dorsal root ganglia [Reymond, et al. 1999] *H-PRUNE* is located in the 1q21.3 chromosomal region, 1Mb upstream the epidermal differentiation complex (EDC), a region which shows a remarkable density of genes that fulfil important functions in terminal differentiation of the human epidermis [Mischke, et al. 1996]. Linkage studies have shown in this region two susceptibility loci for skin inflammation disorders such as psoriasis [Capon, et al. 1999, Hardas, et al. 1996] and atopic dermatitis [Cookson, et al. 2001] for which no genes have been yet identified.

H-Prune participates in a complex network of interactions with proteins involved in cell cycle and cell motility. *In vitro* experiments have shown that h-Prune overexpression increases cellular proliferation in NIH3T3 cells [Forus, et al. 2001]. It has recently been shown that together with GSK-3 $\beta$ , h-Prune cooperatively regulates the disassembly of focal adhesions, to promote cell migration. In-vitro binding studies using these recombinant proteins have demonstrated that GSK-3 $\beta$  binds directly to h-Prune. The aminoacid region spanning from residue 333 to residue 453 of h-Prune is necessary and sufficient for the complex formation with GSK-3 $\beta$  in intact cells. GSK-3 $\beta$  kinase inactive mutants or GSK3 $\beta$  inhibitor treatment decreases the formation of the complex between GSK3 $\beta$  and h-Prune and suppresses cell migration. These results indicate that the kinase activity of GSK-3 $\beta$  is required for its interaction with h-Prune in intact cells [Kobayashi, et al. 2006].

## 1.2. The skin

### 1.2.1. Skin anatomy

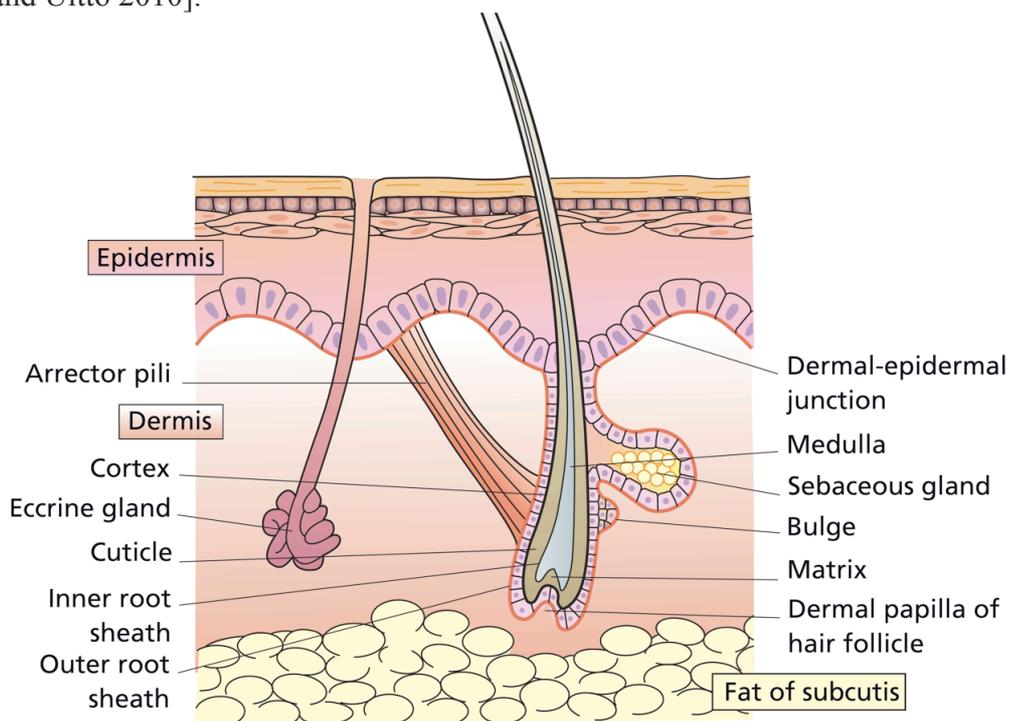
Skin is the largest organ in the body. Human skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue. Beneath the dermis is a layer of subcutaneous fat, which is separated from the rest of the body by a vestigial layer of striated muscle. The epidermis is mainly composed of keratinocytes and is typically 0.05–0.1 mm in thickness. It is formed by division of cells in the basal layer which give rise to the spinous layer. This layer contains cells that move outwards and progressively differentiate, forming the granular layer and the stratum corneum. The cellular progression from the basal layer to the skin surface takes about 30 days but is accelerated in diseases such as psoriasis. The ‘bricklike’ shape of keratinocytes is provided by a cytoskeleton made of keratin intermediate filaments. As the epidermis differentiates, the keratinocytes become flattened. This process involves the filament aggregating protein, filaggrin, a protein component of keratohyalin granules. Indeed, keratin and filaggrin comprise 80–90% of the mass of the epidermis. The outermost layer

of the epidermis is the stratum corneum, where cells (now called corneocytes) have lost nuclei and cytoplasmic organelles. The corneocyte has a highly insoluble, cornified envelope within the plasma membrane, formed by cross-linking of soluble protein precursors, including involucrin and loricrin, the latter contributing 70–85% to the mass of the cornified cell envelope; it also contains several lipids (fatty acids, sterols and ceramides) released from lamellar bodies within the upper living epidermis.

Other cells in the epidermis are the melanocytes, Langerhans' cells and Merkel cells. Melanocytes are dendritic cells that distribute packages of melanin pigment in melanosomes to surrounding keratinocytes to give skin its colour. The number of melanocytes does not differ much between white and black skin. Rather it is the nature of the melanin and the size of the melanosomes that account for the different appearances [Lin and Fisher 2007]. The Langerhans' cells are also dendritic in nature, although these are of mesenchymal origin and originate from bone marrow. Langerhans' cells are antigen-presenting cells and process antigens encountered by the skin to local lymph nodes and thus have a key role in adaptive immune responses in the skin. Merkel cells are probably derived from keratinocytes. They have a role as mechanosensory receptors in response to touch. Human skin contains pilosebaceous follicles and sweat glands. The hair follicles comprise pockets of epithelium that are continuous with the superficial epidermis but which also envelop a small papilla of dermis at their base. A bundle of smooth muscle, the arrector pili, extends at an angle between the surface of the dermis and a point in the follicle wall. Above the insertion, there are holocrine sebaceous glands which open into the pilary canal. In some sites, such as the axillae, the follicles may be associated with apocrine glands. Also derived from the epidermis and opening directly to the skin surface are the eccrine sweat glands.

The epidermis is attached to the dermis via a complex network of proteins and glycoproteins that extend from inside basal keratinocytes into the superficial dermis. Besides adhesion, the dermal–epidermal junction components also contribute to cell migration (for example during wound healing) as well as epithelial–mesenchymal signaling events. Over 30 different macromolecules (collagens, laminins, integrins) interact within a basement membrane zone that is less than 200  $\mu\text{m}$  across. The dermis is a supporting matrix or ground substance in which polysaccharides and proteins are linked to produce macromolecules that have a remarkable capacity for retaining water. The thickness of the dermis varies from less than 0.5 mm to more than 5 mm. There are two principal types of protein fibre: collagen and elastic tissue. Collagen is the major extracellular matrix protein comprising 80–85% of the dry weight of the dermis. Twenty-nine different collagens have been identified in vertebrate tissue (depicted by Roman numerals in the order of their discovery, from I to XXIX), of which at least 12 are expressed in skin. The main interstitial dermal collagens are types I and III whereas the principal basement membrane collagen (at the dermal–epidermal junction and around dermal blood vessels, nerves and appendages) is type IV collagen. Triple-helical collagen monomers polymerize into fibrils and fibres, which then become stabilized by the complex formation of both intra- and intermolecular crosslinks. Collagen fibres are extremely tough and provide skin with its tensile strength. Elastic fibres account for no more than 2–4% of the

extracellular matrix in the dermis and consist of two components, elastin and elastin-associated microfibrils, which together give skin its elasticity and resilience. Elastic microfibrils are composed of several proteins, including fibrillin, which surround the elastin and which can extend throughout the dermis in a web-like configuration to the junction between the dermis and the epidermis. The dermis also contains a number of non-collagenous glycoproteins, including fibronectins, fibulins and integrins. These extracellular matrix components facilitate cell adhesion and cell motility. Between the dermal collagen and elastic tissue is the ground substance made up of glycosaminoglycan/proteoglycan macromolecules. These contribute only 0.1–0.3% of the total dry weight of the dermis but provide a vital role by maintaining hydration, mostly due to the high water-binding capacity of hyaluronic acid. About 60% of the total weight of the dermis is water. The dermis has a very rich blood supply, although no vessels pass through the dermal–epidermal junction. There is a superficial and a deep vascular plexus. The motor innervation of the skin is autonomic, and includes a cholinergic component to the eccrine sweat glands and adrenergic components to both the eccrine and apocrine glands, to the smooth muscle and the arterioles and to the arrector pili muscle. The sensory nerve endings are of several kinds; some are free, some terminate in hair follicles and others have expanded tips [Mc Grath and Uitto 2010].



**Fig 2. The skin and its appendages.** The skin is made up epidermis, dermis and subcutaneous fat layer (subcutis). The epidermis is the thin outer layer of the skin. The dermis is the middle layer of the skin. The dermis contains blood vessels, lymph vessels, hair follicles, sweat glands, collagen bundles, fibroblasts, nerves. The dermis gives skin flexibility and strength. It also contains pain and touch receptors. The subcutis is the deepest layer of skin. It consisting of a network of collagen and fat cells, helps conserve the body's heat and protects the body from injury by acting as a shock absorber. (Modified from Mc Grath & Uitto, *Rook's Textbook of Dermatology*, 2010).

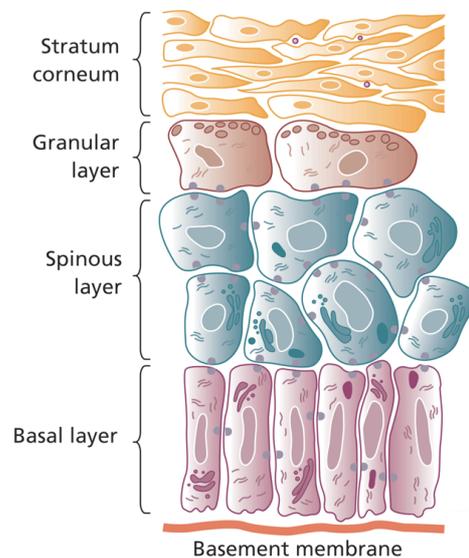
### 1.2.2. Function of Skin

A key role of skin is to provide a mechanical barrier against the external environment. The cornified cell envelope and the stratum corneum restrict water loss from the skin while keratinocyte-derived endogenous antibiotics (defensins and cathelicidins) provide an innate immune defence against bacteria, viruses and fungi. The epidermis also contains a network of about  $2 \times 10^9$  Langerhans' cells, which serve as sentinel cells whose prime function is to survey the epidermal environment and to initiate an immune response against microbial threats, although they may also contribute to immune tolerance in the skin. Melanin, which is mostly found in basal keratinocytes, also provides some protection against DNA damage from ultraviolet radiation. An important function of skin is thermoregulation. Vasodilatation or vasoconstriction of the blood vessels in the deep or superficial plexuses helps regulate heat loss. Eccrine sweat glands are found at all skin sites and are present in densities of 100–600/cm<sup>2</sup>; they play a role in heat control and produce approximately 1 litre of sweat per hour during moderate exercise. Secretions from apocrine sweat glands contribute to body odour (pheromones). Skin lubrication and waterproofing is provided by sebum secreted from sebaceous glands. Subcutaneous fat has important roles in cushioning trauma as well as providing insulation and a calorie reserve. In non-obese subjects, about 80% of the body's total fat is found in subcutaneous tissue. Fat also has an endocrine function, releasing the hormone leptin, which acts on the hypothalamus to regulate hunger and energy metabolism. Other functions of fat cells include tissue remodelling and phagocytosis. Skin also has a key function in synthesizing various metabolic products, such as vitamin D [Mc Grath and Uitto 2010].

### 1.2.3. Epidermal structures

The normal epidermis is a terminally differentiated, stratified, squamous epithelium. The major cell, making up 95% of the total, is the *keratinocyte*, which moves progressively from attachment to the epidermal basement membrane towards the skin surface, forming several well-defined layers during its transit [Houben, et al. 2007]. Thus, on simple morphological grounds, the epidermis can be divided into four distinct layers: *stratum basale* or *stratum germinativum*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. The term *Malpighian* layer includes both the basal and spinous cells. Other cells within the epidermis include melanocytes, Langerhans' cells and Merkel cells. The stratum basale is a continuous layer that is generally only one cell thick but it may be two to three cells thick in glabrous skin and in hyperproliferative epidermis. The basal cells are small and cuboidal (10–14 nm in diameter) and have large, dark staining nuclei, and dense cytoplasm which contains many ribosomes and dense tonofilament bundles. Immediately above the basal cell layer, the epibasal keratinocytes enlarge to form the spinous/prickle-cell layer or *stratum spinosum*. The stratum spinosum is succeeded by the *stratum granulosum* or granular layer, which contains intracellular granules of keratohyalin. At high magnification, the dense mass of keratohyalin granules from human epidermis has a particulate substructure, with particles of irregular shape, on average 2 nm in length, and occurring

randomly in rows or lattices. The cytoplasm of cells of the upper, spinous layer and granular cell layer also contains smaller lamellated granules averaging 100–300 nm in size, which are known as lamellar granules or bodies, membrane-coating granules. These are numerous within the uppermost cells of the spinous layer and migrate towards the periphery of the cells as they enter the granular cell layer. They discharge their lipid components into the intercellular space, playing important roles in barrier function and intercellular cohesion within the stratum corneum [Feingold 2007]. The outermost layer of epidermis is the stratum corneum where cells (now corneocytes) have lost nuclei and cytoplasmic organelles. The cells become flattened and the keratin filaments align into disulphide cross-linked macrofibres, under the influence of *filaggrin*, the protein component of the keratohyalin granule, responsible for keratin filament aggregation. The key role of filaggrin in skin biology has been demonstrated by the discovery of very common loss-of-function mutations in the filaggrin gene as the cause of the genetic disorder ichthyosis vulgaris and as a major risk factor for the development of atopic dermatitis, atopic asthma and systemic allergies [Sandilands, et al. 2006]. The corneocyte has a highly insoluble, cornified envelope within the plasma membrane, formed by crosslinking of the soluble protein precursor, *involucrin*, following the action of a specific epidermal transglutaminase also synthesized in the high stratum spinosum. The process of desquamation involves degradation of the lamellated lipid in the intercellular spaces and loss of the residual intercellular desmosomal interconnections [Milstone 2004]. In palmoplantar skin there is an additional zone, also electron-lucent, the *stratum lucidum*, between the granulosum and corneum. These cells are still nucleated, and may be referred to as ‘transitional’ cells.



**Fig 3. Structure of epidermis.** The epidermis contains 4 layers. From bottom to top the layers are named stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The bottom layer, the stratum basale, has cells that are shaped like columns. In this layer the cells divide and push already formed cells into higher layers. As the cells move into the higher layers, they flatten and eventually die. The top layer of the epidermis, the stratum corneum, is made of dead, flat skin cells that shed about every 2 weeks. (Modified from Mc Grath & Uitto, *Rook's Textbook of Dermatology*, 2010)

#### 1.2.4. Keratinocytes

The keratinocytes are named so, cause the intermediate filaments, that make up the cytoskeleton of this cells, are composed of keratin. The human genome possesses 54 functional keratin genes located in two compact gene clusters, as well as many nonfunctional pseudogenes, scattered around the genome [Schweizer, et al. 2006]. Keratin genes are very specific in their expression patterns. Each one of the many highly specialized epithelial tissues has its own profile of keratin gene expression. Hair and nails express modified keratins, containing large amounts of the amino acid cysteine which forms numerous chemical cross-links to further strengthen the cytoskeleton.

The genes encoding individual keratins fall into two gene families: type I (basic) and type II (acidic). Mapping the tissue distribution of keratins shows coexpression of particular acidic–basic pairs in a cell- and tissue-specific manner. Heterodimers are assembled into higher-order protofibrils and protofilaments by an antiparallel stagger of some complexity. Simple epithelia are characterized by the keratin pair K8/K18, and the stratified squamous epithelia by K5/K14. In addition, stratified squamous epithelia express up to four other keratin pairs during epithelial differentiation. In skin, suprabasal keratins K1/K10 are characteristic of epidermal differentiation. In the stratum granulosum, release of filaggrin from the keratohyalin granules forms macrofibers. Retinoid levels, growth factors and hormones may regulate keratin gene expression. Mesenchymal signals may also direct or permit intrinsic patterns of keratinocyte differentiation. K15 is expressed in basal keratinocytes of the hairfollicle bulge region at the site of pluripotential stem cells. K9 and K2e expression is site restricted in skin: K9 to palmoplantar epidermis and K2e to superficial interfollicular epidermis. Apart from their structural properties, keratins may also have direct roles in cell signaling, the stress response and apoptosis [Magin, et al. 2007]. In epidermal hyperproliferation, as in wound healing and psoriasis, expression of suprabasal keratins K6/K16/K17 is rapidly induced. Currently, 21 of the 54 known keratin genes have been linked to monogenic genetic disorders, and some have been implicated in more complex traits, such as idiopathic liver disease or inflammatory bowel disease [Uitto, et al. 2007].

#### 1.2.5. Skin barrier

One of the key functions of the epidermis is to form a barrier against the external environment. To that end, terminal differentiation of keratinocytes leads to formation of the cornified cell envelope. The cornified envelope is rendered highly insoluble by the formation of glutamyl-lysyl isodipeptide bonds between envelope proteins, catalysed by transglutaminases [Candi, et al. 2005]. *Involucrin* is the best-established envelope precursor

although it is also a marker of differentiation in epithelia such as cornea. Other cytosolic envelope precursors include the family of small proline-rich proteins (SPR1) including *cornifin* or SPR1 and *pancornulins*. Other envelope proteins include *SKALP/elafin* and *keratolinin/cystatin*, a 36-kDa protein cross-linked by transglutaminase into a 150-kDa multimer. Some precursors of the cornified envelope are delivered by granules. Small, smooth, sulphur-rich L granules contain the cysteine-rich protein *loricrin*, and accumulate in the stratum granulosum [Ishida-Yamamoto, et al. 1999].

Loricrin is the major component of the cornified envelope. Expression of loricrin depends on a high calcium concentration and is down-regulated by retinoids. Loricrin gene expression is limited to terminally differentiating keratinocytes of stratified squamous epithelia. Due to the characteristic pattern expression of this protein, its promoter is often used to drive the expression of transgenes in the epidermis. In the 1999 was created a modified Loricrin promoter (ML) that was able to drive the transgene expression in all four layer of epidermis [DiSepio, et al. 1999]. The ML promoter was extensively used to obtain specific expression of transgene in the entire epidermis. In the 1999 Waikel and colleagues used this promoter upstream Human c-Myc cDNA, obtained an overexpression of the oncogene in the entire epidermis. However the expression described in the work was not lasting: ML-myc2 pups exhibit a severe hyperkeratotic phenotype, which develops on day 4. The phenotype persists for several more days and then the pups begin to normalize [Waikel, et al. 1999].

Profilaggrin in F granules may make a minor contribution to the envelope. Membrane-associated proteins that contribute to the cornified envelope include the plakin family members, periplakin, envoplakin, epiplakin, desmoplakin as well as plectin. Formation of the cornified cell envelope is triggered by a rise in intracellular calcium levels [Bikle, et al. 2004]. This leads to cross-link formation between plakins and involucrin catalysed by transglutaminase. Other desmosomal proteins are then also cross-linked, forming a scaffold along the entire inner surface of the plasma membrane. Ceramides from the secreted contents of lamellar bodies are then esterified onto glutamine residues of the scaffold proteins. The cornified cell envelope is reinforced by the addition of a variable amount of SPRs, repetin, trichohyalin, cystostatin  $\alpha$ , elafin and LEP/XP-5 (skin-specific protein). Although most desmosomal components are degraded, keratin intermediate filaments (mostly K1, K10 and K2e) may be cross-linked to desmoplakin and envoplakin remnants. Together these assembly and degradation events result in durable, flexible but dead cells that have vital mechanical and water-permeability barrier functions.

During epidermal differentiation, there are profound changes in the composition of lipids; in the viable layers phospholipids, cholesterol and triglycerides predominate, but in the upper stratum spinosum and stratum granulosum lipid is synthesized and packaged into lamellated, membrane-bound organelles known as *membrane-coating granules*, lamellar granules or Odland bodies [Elias, et al. 2008]. They are found adjacent to the cell membrane with alternating thick and thin dense lines separated by lighter lamellae of equal width, consistent with packing of flattened discs within a membrane boundary. Immunocytochemistry has confirmed the presence of phospholipids, glycolipids and free sterols. The organelles move towards the

plasma membrane as the cells move through the granular layer and cluster at the cell membrane. They fuse with the plasma membrane, dispersing their contents into the intercellular space. Polar lipids from the lamellar granules are remodelled into neutral lipids in the intercellular space between corneocytes, forming an important barrier to permeability. The lamellar granules also contain hydrolytic enzymes, lipases and glycosidases, which are responsible for this remodelling. Thus, the stratum corneum is rich in ceramides, free sterols and free fatty acids. Linoleate appears to be essential for proper barrier function as an ester-linked residue in acyl ceramides; thus, in essential fatty acid deficiency, substitution of this linoleate by oleate causes a defect in barrier function. The skin is an active lipid-synthesizing tissue, with a daily production rate of 100 mg/day. In human epidermis, nearly all sterol is present as cholesterol. In many cells, cholesterol biosynthesis may be modulated by uptake of exogenous cholesterol, mediated by low-density lipoprotein (LDL) receptors, but these are only present on the plasma membrane of basal keratinocytes, and are rapidly down-regulated on epidermal differentiation. Sterol synthesis is higher in the stratum granulosum, and is thus relatively autonomous and uninfluenced by dietary or circulating sterol levels. Cholesterol sulphate is highest in the stratum granulosum and corneum, where it is thought to play a role in cell cohesion.

#### **1.2.6. Skin maintenance**

Responsible of maintaining skin homeostasis, regenerate skin appendages and repair itself after injury, are the stem cells which reside in the bulge area of hair follicles, the basal layer of interfollicular epidermis and the base of sebaceous glands [Braun and Prowse 2006]. These stem cells generate a proliferative progeny that can undergo differentiation. Stem cells are able to self-renew as well as give rise to the differentiating cells [Fuchs 2008]. In the epidermis, some basal cells can periodically withdraw from the cell cycle and commit to terminal differentiation. It is still not clear, however, whether every cell or only a proportion of cells in the basal layer is a stem cell. One long-established theory divides basal keratinocytes into epidermal proliferation units, which comprise one self-renewing stem cell and about 10 tightly packed, transient, amplifying cells (each capable of dividing several times and then exiting the basal layer to undergo terminal differentiation) [Potten 1974]. This unit gives rise to a column of larger and flatter cells that culminates in a single hexagonal surface. Stem cells within epidermal proliferation units are associated with a profile of particular chemical, molecular and biological characteristics [Watt and Hogan 2000]. For example, stem cells retain labelling with injected <sup>3</sup>H-thymidine or Brdu after repeated cell division. In culture, actively growing clones present after serial passaging are considered to indicate an origin from stem cells. Potential markers of interfollicular epidermal stem cells are  $\alpha 6$  and  $\beta 1$  integrin as well as p63, whereas sebocyte stem cells express Blimp1. Markers of hair follicle bulge stem cells include CD34, NFATc1, vitamin D receptor, TCF3, Sox9 and Lhx2. In the epidermal proliferation unit concept of stem cell behaviour, the division of basal cells has been viewed as a symmetrical process in which equal daughter cells are generated; the basal cells progressively reduce their

adhesiveness to the underlying epidermal basement membrane, delaminate and commit to terminal differentiation. However, recent data suggest that basal cells can also undergo asymmetrical cell division, shifting their spindle orientation from lateral to perpendicular [Lechler and Fuchs 2005]. Asymmetrical cell divisions provide a natural means of maintaining a proliferative daughter cell that retains the cell markers associated with stem cells, while the other daughter cell has reduced markers such as  $\beta 1$  integrin, increased expression of Notch signals, and is committed to terminal differentiation. Asymmetrical cell divisions, therefore, can bypass the need for transient amplifying cells. The structural and biological composition of the dermal–epidermal junction also influences the proliferative properties of basal keratinocytes. Laminin-332 promotes anchorage as a ligand via  $\alpha 6\beta 4$  integrins in hemidesmosomes and signaling/migration via its association with  $\alpha 3\beta 1$  in focal adhesions. Signaling via  $\alpha 3\beta 1$  integrin stimulates the MAP-kinase pathway, turnover of focal adhesions and epidermal migration. The basement membrane is also a reservoir for growth factors that can promote epidermal proliferation (e.g. TGF- $\alpha$ , EGFs and insulin growth factors) or restrict it (e.g. TGF- $\beta$ ). EGFR signaling also enhances proliferation and migration in the epidermis, possibly by phosphorylating  $\beta 4$  integrin and promoting hemidesmosome disassembly. Thus the control of basal keratinocyte stem cell activity in maintaining homeostasis and responding to injury is through the regulation of at least two opposing tyrosine kinase pathways and two integrin structures.

A key transcription factor in regulating the self-renewal and long-term proliferative capacity of the stem cell is p63, a member of the p53 family of proto-oncogenes. However, the precise role of p63 is not clear; it may have a direct effect on stem-cell renewal, or lineage commitment, and/or an effect on switching from proliferation to terminal differentiation [Truong and Khavari 2007].

Stem cells in hair follicles are located in the lowest permanent part of the follicle, within the outer root sheath. These cells cycle more slowly than other cells and have the capacity to migrate (e.g. to the base of the hair follicle in follicular regeneration), as well as to differentiate into diverse lineages (e.g. outer root sheath, inner root sheath, hair shaft, sebocytes and interfollicular epidermis). Despite this multipotency, however, the follicle stem cells only function in pilosebaceous unit homeostasis and do not contribute to interfollicular epidermis unless the skin is wounded [Ito, et al. 2007]. Hair follicles undergo cycles of degeneration and regeneration throughout life. During the growth phase (anagen), which requires activation of hair follicle stem cells, matrix cells proliferate rapidly but then undergo sudden apoptosis (catagen). The hair bulb and root shrivel to form an epithelial strand which forces the dermal papilla to rest at the base of the non-cycling part of the hair follicle [Cotsarelis 2006]. The hairs then enter a resting phase (telogen). At a molecular level, inhibition of BMP signaling and activation of Wnt signaling converge to regulate stem cell activation. From microarray studies, *Sox9*, *Tcf3* and *Lhx2* appear to be markers of follicular stem cells whether they are quiescent or proliferative.

### 1.3. Wnt signaling

Wnts are a family of secreted, cysteine-rich, glycosylated, protein ligands that influence cell growth, differentiation, migration and fate [Miller 2002, Polakis 2000, Smalley and Dale 1999]. Members of Wnt family have been identified in organisms ranging in complexity from the cnidarian Hydra to humans; at least 19 different Wnts exist in mammals [Miller 2002]. Severe developmental defects usually occur in mice that have defective Wnt signaling.

One of the pathways regulated by Wnt molecules is termed “canonical Wnt pathway” or “Wnt/ $\beta$ -catenin pathway” [Huelsenken and Behrens 2002, Polakis 2000, Seidensticker and Behrens 2000, Sharpe, et al. 2001]. In the canonical Wnt pathway, Wnt signal transduction ultimately results in the activation of genes regulated by the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of architectural transcription factors [Barker, et al. 2000, Brantjes, et al. 2002, Novak and Dedhar 1999].

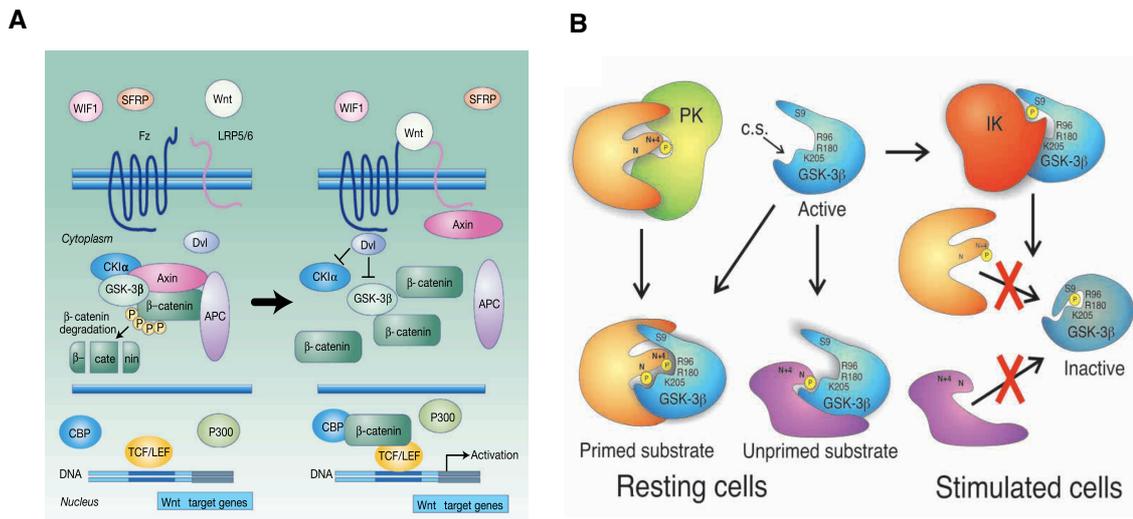
The effector molecule responsible for activating TCF/LEF-responsive genes is  $\beta$ -catenin, which serves as a transactivator that binds to DNA bound TCF/LEFs. As in insulin signaling, GSK-3 $\beta$  plays a key inhibitory role in the Wnt pathway. In unstimulated cells, GSK-3 $\beta$  phosphorylates the N-terminal domain of  $\beta$ -catenin, thereby targeting it for ubiquitylation and proteasomal degradation. Exposure of cells to Wnts leads to inactivation of GSK-3 $\beta$  through an as yet unclear mechanism. The phosphoprotein Dishevelled is required, after receptor-ligand interaction, to transduce the signal that results in the inactivation of GSK-3 $\beta$ . As a result,  $\beta$ -catenin is dephosphorylated [van Noort, et al. 2002] and escapes the ubiquitylation-dependent destruction machinery. Unphosphorylated  $\beta$ -catenin accumulates in the cytoplasm and translocates to the nucleus, where it can associate with the TCF/LEFs and become a transcriptional transactivator. Mutations in  $\beta$ -catenin that prevent its phosphorylation by GSK-3 $\beta$  (gain-of-function mutations) have been found in cancers of the skin, colon, prostate, liver, endometrium and ovary [Polakis 2000].

Many components of the Wnt pathway, and their functional roles, were first identified in studies of Wnt pathway in *Drosophila melanogaster*, which is driven by the Wnt homologue, Wingless [Dierick and Bejsovec 1999, Manoukian and Woodgett 2002]. In *Drosophila*, the GSK-3 $\beta$  homologue is termed Shaggy [Bourouis, et al. 1990] or Zeste-white3 [Siegfried, et al. 1990]. Phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$  occurs in a complex sometimes referred as “destruction complex”, which consists minimally of the proteins GSK-3,  $\beta$ -catenin, axin/conductin and adenomatous polyposis coli (APC) [Hinoi, et al. 2000]. APC is a tumour suppressor protein commonly deleted in familial adenomatous polyposis and sporadic colorectal cancer [Polakis 1997]. Axin (and a related protein known as conductin or axil) harbours several protein-protein interaction domains and serves as a scaffolding protein that holds together the elements of the  $\beta$ -catenin destruction complex. Both Axin and APC are phosphorylated by GSK-3 $\beta$ . Phosphorylation of axin by GSK-3 $\beta$  increases its stability and binding to  $\beta$ -catenin [Yamamoto, et al. 1999]. Phosphorylation of APC increases its binding to  $\beta$ -catenin

[Rubinfeld, et al. 1996]. It was determined that  $\beta$ -catenin is also a primed substrate for GSK-3 $\beta$ , with casein kinase I (CKI) acting as the priming kinase [Amit, et al. 2002]. CKI phosphorylates S45, which lies four residues C-terminal to three GSK-3 $\beta$  targets at serines S33, S37 and S41. As a priming kinase, CKI functions as a negative regulator of Wnt signaling since it promotes GSK-3 $\beta$  function. CKI binds to axin and Dishevelled and phosphorylates not only  $\beta$ -catenin but also axin, Dishevelled and APC. Stimulation of cells with insulin causes inactivation of GSK-3 $\beta$  through a phosphoinositide 3-kinase (PI 3 kinase)-dependent mechanism. PI-kinase-induced activation of PKB (also termed Akt) results in PKB phosphorylation of both GSK-3 $\beta$  isoforms (S9 of GSK-3b; S21 of GSK-3a) [Cross, et al. 1995], inhibiting its activity. This leads to the dephosphorylation of substrates including glycogen synthase and eukaryotic protein synthesis initiation factor-2B (eIF-2B), resulting in their functional activation and consequent increasing of glycogen and protein synthesis [Cohen, et al. 1997].

Numerous other stimuli also lead to inactivation of GSK-3 $\beta$  through S9/S21 phosphorylation, including growth factors such as EGF and PDGF that stimulate the GSK-3 $\beta$ -inactivating kinase p90RSK (also known as MAPKAP-K1) through MAP kinases [Brady, et al. 1998], activators of p70 ribosomal S6 kinase (p70S6K) such as amino acids [Terruzzi, et al. 2002], activators of cAMP-activated protein kinase (PKA) [Tanji, et al. 2002] and PKC activators [Fang, et al. 2002]. Insulin induces an increased activity of glycogen synthase but has no influence on the protein level of  $\beta$ -catenin. In contrast, Wnt increases the cytosolic pool of  $\beta$ -catenin but not glycogen synthase activity. Unlike insulin, neither the phosphorylation status of the serine9 residue of GSK3 $\beta$  nor the activity of protein kinase B is regulated by Wnt. Although the decrease in GSK3 $\beta$  activity is required, GSK3 $\beta$  is not a limiting component for Wnt signaling in the cells. The axin-conductin complexed GSK3 $\beta$  is dedicated to Wnt rather than insulin signaling. Insulin and Wnt pathways regulate GSK3b through different mechanisms, and therefore lead to distinct downstream events [Ding, et al. 2000].

An increase in *Wnt*/ $\beta$ -catenin signaling is likely to be biologically significant, as it has been associated with maintenance of keratinocytes in their stem cell compartment [Zhu and Watt 1999] and a number of malignancies, including hair follicles tumor formation [Gat, et al. 1998]. Efforts to understand how the keratin genes are differentially regulated led to the discovery that hair shaft *Ha/Hb* keratin genes are genuine Wnt target genes, and that Wnt signaling has a crucial role in the differentiation of matrix cells along a hair shaft lineage [Merrill, et al. 2001]. Finally the Wnt is one of most important pathway involved in the follicle stem cell activation: stabilization of  $\beta$ -catenin promotes anagen and hair cycle progression [Fuchs 2007]. Wnt deregulation leads to an imbalance of proliferation and differentiation, often resulting in cancers [Reya, et al. 2001].  $\beta$ -catenin controls lineage selection of epidermal stem cells: high levels of  $\beta$ -catenin activation favours hair follicle differentiation, whereas inhibition of activation favours interfollicular epidermal differentiation [Fuchs, et al. 2004, Niemann and Watt 2002]



**Fig 4. Canonical Wnt pathway (A) and GSK3  $\beta$  regulation (B).** (A) Protein complex formed by axin, APC, GSK-3 $\beta$ , and CK1 $\alpha$  (named  $\beta$ -catenin destruction complex) binds to  $\beta$ -catenin in the absence of Wnt, resulting in the degradation of  $\beta$ -catenin by 26S proteasome system. WIF1 and sFRPs can work as endogenous inhibitors of the Wnt signaling pathway (left). When Wnt binds to the receptor Fz and co-receptor LRP5/6, these receptors mediate signals into cells and inhibit the function of  $\beta$ -catenin destruction complex. Accumulated- $\beta$ -catenin in the cytoplasm translocates to nucleus. In the nucleus,  $\beta$ -catenin activates the transcription of target genes together with transcription factors (TCF/LEF) and other transcriptional cofactors, such as CBP. (Taken from Takahashi-yanga e Kahn, *Clinical Cancer Research*, 2010). (B) Regulation of GSK-3 $\beta$  activity by serine phosphorylation. In the resting cell, GSK-3 $\beta$  is constitutively active. Both unprimed substrates and substrates phosphorylated by a priming kinase (PK) are capable of being phosphorylated by the active GSK-3 $\beta$ . The priming phospho-residue at position N + 4, binds a pocket of positive charge arising from the arginine (R) and lysine (K) residues indicated. This directs a serine or threonine at position N to the active catalytic site (C.S.). When an inactivating kinase (IK) such as PKB/Akt phosphorylates GSK-3 $\beta$  on serine 9 (S9), the phosphorylated Nterminus becomes a primed pseudo-substrate that occupies the positive binding pocket and active site of the enzyme, acting as a competitive inhibitor for true substrates. This prevents phosphorylation of any substrates. (Modified from Bradley and Woodgett, *journal of cell science* 2003)

#### 1.4.GSK 3 $\beta$

Glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) is a serine/threonine kinase that was first isolated and purified as an enzyme capable of phosphorylating and inactivating the enzyme glycogen synthase [Embi, et al. 1980, Woodgett and Cohen 1984]. GSK-3 $\beta$  acts as a downstream regulatory switch that determines the output of numerous signaling pathways initiated by diverse stimuli. GSK-3 $\beta$  regulates several physiological responses in mammalian cells, including protein synthesis, gene expression, subcellular localization of proteins, and protein degradation, in mammalian cells by phosphorylating many substrates [Frame and Cohen 2001, Grimes and Jope 2001, Woodgett 2001].

The pathways in which GSK-3 $\beta$  acts as a key regulator, when dysregulated, have been implicated in the development of human diseases such as diabetes, Alzheimer's disease, bipolar disorder and cancer. Evidence that GSK-3 $\beta$  regulates cellular architecture in neuronal cells has been accumulated [Cohen and Frame 2001, Jope and Johnson 2004]. Two microtubule-associating proteins, Tau and MAP1B, are phosphorylated by GSK-3 $\beta$ , which regulates their binding to microtubules, thereby modulating microtubule dynamics. An inactive pool of GSK-3 $\beta$  has been found to be localized at the leading edge of the cells alongside F-actin, and semapholin 3A and lysophosphatidic acid activate GSK-3 $\beta$ , causing growth cone collapse and neurite retraction [Eickholt, et al. 2002]. GSK-3 $\beta$  mediates Par6-PKC $\zeta$ -dependent promotion of polarization and cell protrusion in astrocytes [Etienne-Manneville and Hall 2003]. Furthermore, GSK-3 $\beta$  phosphorylates CRMP2 to specify the fate of axons and dendrites [Yoshimura, et al. 2005]. GSK-3 $\beta$  has also been shown to be involved in signaling activated by cell adhesion in nonneuronal cells [Ouatras, et al. 2003, Roberts, et al. 2004]. The formation of extending lamellipodia in migrating keratinocytes is blocked by GSK-3 $\beta$  inhibitors [Koivisto, et al. 2003]. The initiation and stimulation of sperm motility are accompanied by the inactivation of GSK-3 $\beta$  [Somanath, et al. 2004]. Although these results suggest that GSK-3 $\beta$  is involved in the dynamics of actin filaments and microtubules, how the GSK-3 $\beta$  activity is linked to molecules involved in cell migration is not clearly understood.

In literature there is more than one evidence of a crosstalk regulation between the Wnt signaling and other pathways. In particular, *Wnt* signaling is suppressed by Notch1 activation, and is elevated in keratinocytes and tumors as a consequence of loss of Notch1 function [Nicolas, et al. 2003].

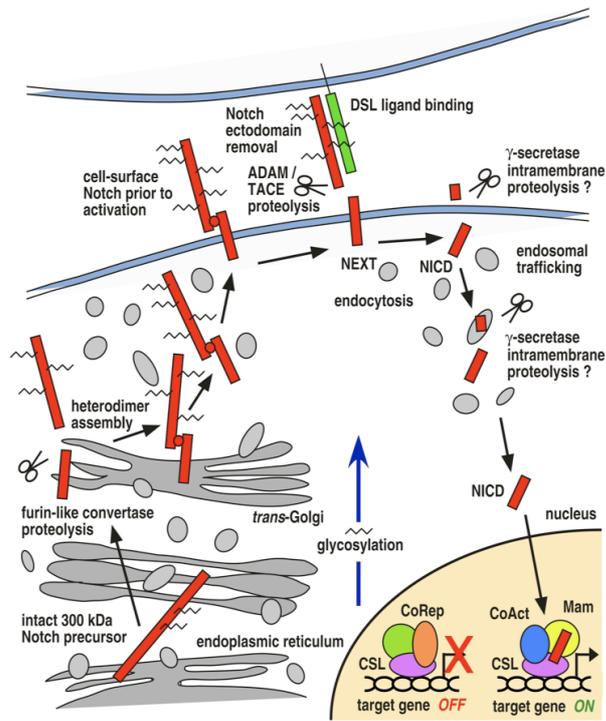
### **1.5. Notch signaling**

Notch signaling controls numerous cell-fate specification events in multicellular organisms, and dysregulated Notch signaling causes several diseases with underlying developmental defects. In vertebrates, Notch signaling has been implicated in many such patterning events as diverse as inner ear hair cell formation, insulin secreting pancreatic  $\beta$  cell production, specification of crypt and goblet cells in the intestine, and multiple steps of B and T cell development within the immune system [Apelqvist, et al. 1999, Lanford, et al. 1999, Robey, et al. 1996, van Es, et al. 2005, Washburn, et al. 1997]. Proper tissue development also requires that fine-scale cellular differentiation is coordinated with the overall control of tissue size and identity, and Notch signaling is widely implicated in many fundamental regulatory processes, including cell proliferation, apoptosis, and the epithelial-mesenchymal transition in higher eukaryotes.

Indeed, it might not be an exaggeration to suggest that nearly all cells of complex animal tissues could potentially require Notch signaling at some point or points in their lineage histories for proper final differentiation. Given the wide-ranging importance of Notch signaling during

animal development, it is not surprising that mutations in genes encoding Notch signaling components have been implicated in several human diseases involving aberrant cellular differentiation or tissue development, including T cell acute lymphoblastic leukemia (T-ALL), Alagille syndrome, spondylocostal dysostosis, tetralogy of Fallot, CADASIL syndrome, and aortic valve disease [Artavanis-Tsakonas, et al. 1999, Gridley 2003]. In such contexts Notch signaling most typically controls the fine cellular patterning and allocation of different cell types within a tissue. Accordingly, Notch signaling depends upon direct contact between the interacting cells. Furthermore, as Notch-dependent patterning events often occur over relatively brief developmental time spans, signal transmission must be both rapid and highly responsive to downregulation and reactivation. The core molecular features of Notch signaling as well as its regulation by a host of posttranslational processes allow the pathway to operate with exquisite spatiotemporal sensitivity and versatility in a wide variety of developmental contexts.

The term “Notch signaling” is generally understood to refer to a specific molecular mechanism that is highly conserved among many organisms and is the best characterized mode of Notch signaling. This mechanism is also referred to as “canonical Notch signaling”, to distinguish it from some atypical signaling modes that have also been documented. Canonical Notch signaling involves activation of Notch receptor at cell surface by ligands of the DSL family, which includes Delta and Serrate/Jagged in *Drosophila* and mammals as well as LAG-2 in *C. elegans*. Members of both Notch receptor and DSL ligand families are, for the most part, type I single-pass integral membrane proteins with extracellular domains consisting primarily of up to 36 tandem EGF-like repeats [Wharton, et al. 1985, Yochem and Greenwald 1989]. Receptor-ligand interactions involve direct binding of an N-terminal ligand domain to the EGF-like repeat 11-12 region of Notch [Rebay, et al. 1991]. Notch signal transduction is initiated by ligand binding and endocytosis, which generate the forces needed to expose an otherwise inaccessible cleavage site in the extracellular portion of the Notch C-terminal fragment. Cleavage at this site produces the activated, membrane-anchored Notch form termed Notch extracellular truncation (NEXT). NEXT is subsequently cleaved by the intramembrane aspartyl protease complex  $\gamma$ -secretase, leading to release of the Notch intracellular signal-transducing fragment termed Notch intracellular domain (NICD) [Bray 2006, Gordon, et al. 2007]. This cleavage can occur at the cell surface and within the endosomal trafficking pathway. In the absence of NICD, most Notch target genes are maintained in an actively repressed state through the formation of transcriptional complexes involving CSL transcription factors and various corepressors (CoRep). NICD possesses nuclear localization sequences [Stifani, et al. 1992] and enters into the nucleus where it participates directly in the transcriptional regulation of target genes [Struhl and Adachi 1998]. Upon nuclear translocation of NICD, corepressors associated with CSL are displaced and a transcriptionally active complex consisting of CSL, NICD, Mastermind (Mam), and coactivators (CoAct) assembles, leading to activation of Notch target genes.



**Fig 5. Notch Receptor Synthesis and Activation.** The Notch receptor is synthesized as a 300 kDa precursor that is processed and glycosylated in the Golgi, until his exposure on the cell surface. Notch signal transduction is initiated by ligand binding and endocytosis, which generate the forces needed to expose an otherwise inaccessible ADAM10/TACE/Kuz/SUP-17 cleavage site in the extracellular portion of the Notch C-terminal fragment. Cleavage at this site produces the activated, membrane-anchored Notch form termed Notch extracellular truncation (NEXT). NEXT is subsequently cleaved by the intramembrane aspartyl protease complex  $\gamma$ -secretase, leading to release of the Notch intracellular signal-transducing fragment termed Notch intracellular domain (NICD). This cleavage can occur at the cell surface and within the endosomal trafficking pathway. In the absence of NICD, most Notch target genes are maintained in an actively repressed state through the formation of transcriptional complexes involving CSL transcription factors and various co-repressors (CoRep). Upon nuclear translocation of NICD, corepressors associated with CSL are displaced and a transcriptionally active complex consisting of CSL, NICD, Mastermind (Mam), and co-activators (CoAct) assembles, leading to activation of Notch target genes. This schematic presents a simplified overview of the main conserved features of Notch synthesis and signaling; details of the biochemical mechanisms involved are omitted for the sake of clarity, the positions of the Notch diagrams are not intended to accurately depict the topology of Notch in various membrane compartments, and the glycosylation symbols and transcriptional complex diagrams are illustrative and do not imply specific glycosylation site locations or protein-protein interactions. (Taken from Fortini, *Cell* 2011).

The rapidly changing levels of pathway activity require that the nuclear effectors do not have a long half-life. This is achieved by recruitment of factors such as cyclin-dependent kinase-8 (CDK8), which phosphorylates NICD, rendering it into a substrate for the nuclear ubiquitin ligase SEL10 [Fryer et al., 2002, Fryer et al., 2004]. In mammalian cells, SEL10

preferentially interacts with a phosphorylated form of NICD and the expression of a dominant negative SEL10 leads to increased expression of Notch targets [Gupta-Rossi, et al. 2001, Oberg, et al. 2001]. This interaction requires the C-terminal PEST region, consistent with observations that *Notch* with C-terminal truncations behave as gain-of-function alleles, and in humans they contribute to oncogenicity [Weng, et al. 2004]. The best characterized Notch targets are the bHLH genes of the HES/HEY families, exemplified by the E(spl) genes in *Drosophila* and HES1 in mouse. Their expression is usually transient and reflects the dynamic nature of Notch signaling. In addition there is evidence for autoregulation such that oscillations of HES expression have been observed and are thought to contribute to clocks that regulate somitogenesis, limb segmentation, and neural progenitor maintenance [Brend and Holley 2009, Kageyama, et al. 2007, Lewis, et al. 2009, Shimojo, et al. 2008]. Altogether HES/HEY have now been shown to function downstream of Notch in many critical processes and to contribute to oncogenesis. All HES/HEY proteins appear to function as transcriptional repressors.

In the human epidermis, Notch1 is expressed in all the epidermal layers; Notch2 is expressed in the basal layer. In the mouse epidermis, however, Notch1 is expressed most strongly in the spinous layer, and Notch2 is also expressed in the spinous layer but not in the basal layer [Blanpain, et al. 2006]. Notch ligands such as Jagged and Delta-like family members are expressed in overlapping patterns with Notch in the epidermis both of humans and mice [Rangarajan, et al. 2001].

Notch1 signaling in mouse keratinocytes stimulates expression of early differentiation markers such as keratin1 and involucrin. This induction is consistent with the epidermal phenotype observed in mice that have constitutively activated Notch1 in the epidermis. Histologically, the Notch1 activated mice show acanthosis, corresponding to an increase in the differentiating (keratin1 positive) cell compartment of the epidermis [Blanpain, et al. 2006]. Conversely a decrease in the differentiating cell compartment is observed in mice that have a keratinocyte specific deletion of the Notch1 gene [Rangarajan et al., 2001]. A substantial increase in the number of layers of proliferating cells occurs in the Notch1 deficient epidermis. Recent studies show that Notch signaling determines spinous cell fate and induces terminal differentiation by a mechanism independent of Hes1, but Hes1 is required for maintenance of the immature state of spinous cells. Notch signaling induces *Ascl2* expression to promote terminal differentiation, while simultaneously repressing *Ascl2* through Hes1 to inhibit premature terminal differentiation. A Notch1/Notch2 double knock out mice model at embryonal stage E18.5 shows a thinner epidermal phenotype: K10-positive suprabasal layer cells were selectively reduced indicating that Notch signaling is required for the generation and/or maintenance of suprabasal cells during epidermal development. K15, a marker for basal layer cells at this stage of epidermal development, was exclusively expressed in basal layer cells. By contrast, the N1N2 dcKO epidermis shows a pronounced number of K15-positive/K10-negative cells in the lowermost suprabasal layer, suggesting retardation of spinous cell differentiation. Furthermore, a significant reduction in loricrin expression was observed in the N1N2 dcKO epidermis, indicating an important role of Notch signaling in granular cell differentiation [Moriyama, et al. 2008].

First, Notch has been found to directly regulate genes involved in proliferation and apoptosis. For example a direct targets of Notch involved in promoting proliferation include CyclinD [Jeffries, et al. 2002, Joshi, et al. 2009], string/CDC25 [Krejci, et al. 2009, Palomero, et al. 2006], and CDK5 [Palomero, et al. 2006]. The keratinocyte-specific deletion of the Notch1 gene results in marked epidermal hyperplasia and deregulated expression of multiple differentiation markers. In differentiating primary keratinocytes in vitro endogenous Notch1 is required for induction of p21WAF1/Cip1 expression, and activated Notch1 causes growth suppression by inducing p21WAF1/Cip1 expression. Activated Notch1 also induces expression of “early” differentiation markers, while suppressing the late markers like loricrin and filaggrin. Induction of p21WAF1/Cip1 expression and early differentiation markers occur through two different mechanisms. The RBP-Jk protein binds directly to the endogenous p21 promoter and p21 expression is induced specifically by activated Notch1 through RBP-Jk-dependent transcription. Expression of early differentiation markers is RBPJk-independent and can be induced by activated Notch1 and Notch2, as well as the highly conserved ankyrin repeat domain of the Notch1 cytoplasmic region. Thus, Notch signaling triggers two distinct pathways leading to keratinocyte growth arrest and differentiation [Rangarajan, et al. 2001]. Phosphorylated forms of NICD1 have been identified within the nucleus [Redmond, et al. 2000, Shimizu, et al. 2000] and have been correlated with CSL members [Foltz and Nye 2001, Kidd, et al. 1998], as well as correlated with regions of the receptor that are required for activity [Foltz and Nye 2001, Ingles-Esteve, et al. 2001].

Genetic studies have suggested that the *Drosophila* homolog of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), Shaggy, may act as a positive modulator of the Notch signaling [Romain, et al. 2001, Ruel, et al. 1993]. Foltz and colleagues have shown that GSK-3 $\beta$  was able to bind and phosphorylate NICD1 in vitro, and attenuation of GSK3 $\beta$  activity reduced phosphorylation of NICD1 in vivo. Functionally, ligand-activated signaling through the endogenous Notch1 receptor was reduced in GSK3 $\beta$  null fibroblasts, implying a positive role for GSK3 $\beta$  in mammalian Notch signaling. As a possible mechanistic explanation of the effect of GSK3 $\beta$  on Notch signaling, they observed that inhibition of GSK3 $\beta$  shortened the half-life of NICD1. Conversely, activated GSK3 $\beta$  reduced the quantity of NICD1 that was degraded by the proteasome. These studies reveal that GSK3 $\beta$  modulates Notch1 signaling, possibly through direct phosphorylation of the intracellular domain of Notch, and that the activity of GSK3 $\beta$  protects the intracellular domain from proteasome degradation.

Notch signaling has an important gate keeper function in the transition from basal to suprabasal cells of epidermis; in mammalian cells, Notch activation is generally thought to maintain stem cell potential and inhibit differentiation, thereby promoting carcinogenesis [Artavanis-Tsakonas, et al. 1999]. However, in specific cell types such as keratinocytes, increased Notch activity causes exit from the cell cycle and commitment to differentiation [Lowell, et al. 2000, Nickoloff, et al. 2002, Rangarajan, et al. 2001]. In mouse keratinocytes, the gene for the cyclin/CDK inhibitor p21WAF1/Cip1 is also induced by the Notch/RBP-Jk complex through both a direct and indirect

mechanism [Mammucari, et al. 2005, Rangarajan, et al. 2001], with p21WAF1/Cip1 functioning downstream of Notch, as a negative transcriptional regulator of *Wnt4* expression [Devgan, et al. 2005]. Notch activation also exerts effects on other pathways important to keratinocyte growth and differentiation; it induces NF- $\kappa$ B [Nickoloff, et al. 2002] and inhibits AP-1 [Chu, et al. 2002] and  $\beta$ -catenin signaling [Devgan, et al. 2005, Nicolas, et al. 2003]. Biologically, both Notch1 [Lowell, et al. 2000] and p21 [Topley, et al. 1999] have been reported to promote the commitment of keratinocyte stem cell populations to differentiation [Missero, et al. 1996, Nicolas, et al. 2003, Topley, et al. 1999]. Beside p21, Notch signaling affects other pathways with significant regulatory functions in keratinocyte growth and differentiation [Lefort and Dotto 2004]. Moreover, Notch1 signaling was found to activate caspase 3, thereby leading to a decrease in proliferation and an increase in the differentiation of embryonic keratinocytes through activation of PKC- $\delta$  [Okuyama, et al. 2004]. Recent work on Notch shows that Notch1 gene expression and activity are substantially down-modulated in keratinocyte cancer cell lines and tumors, and that Notch1 gene is a p53 target in human keratinocytes with a role in tumor suppression [Lefort, et al. 2007]. Indeed in mouse model ablation of *Notch1* results in epidermal and corneal hyperplasia followed by the development of skin tumors and facilitated chemical-induced skin carcinogenesis.

## 1.6.Psoriasis

Psoriasis is a relatively common skin disease affecting 1% to 2% of the population the main feature of psoriasis is a red scaly area or patch. The patches appear particularly on the knees, elbows and scalp and sometimes on other parts of the trunk, and legs. Histological the chronic lesions demonstrate epidermic hyperplasia, parakeratosis, desquamation and small abscesses of neutrophils in epidermis. Types of psoriasis include plaque, guttate, erythrodermic and pustular [Peters, et al. 2000]. Males and females are equally affected and 75% of patients develop the disease before the age of 40. First manifestations of the disease are most common in the third decade. Two peaks of age of onset have been described: one at 20–30 years and a smaller peak at 50–60 years. Psoriasis can also occur with other inflammatory diseases such as (psoriatic) arthritis in 10–30%. Psoriasis also occurs in association with human immunodeficiency virus (HIV) infection. It is hypothesized that psoriasis is due to a combination of genetic predisposition and environmental assaults. These can include injury, infection, stress or certain medications.

The pathogenesis of inflammatory diseases such as psoriasis involve a large number of deregulated genes and gene clusters, particularly those involved in epithelial proliferation and in the immune system. However, these studies have not provided sufficient insights to lead to an identification of the molecular defects underlying the disease. The normal cycle of maturation of keratinocytes is 28–30 days. In psoriasis this is accelerated to 3–4 days. The immune system has been strongly implicated in the pathogenesis of psoriasis since it resembles a T cell-mediated autoimmune

disease [Gottlieb 2001]. During lesion formation, inflammation precedes epidermal hyperproliferation and increased numbers of T cells have been demonstrated in the uninvolved skin of psoriatic patients [Baadsgaard, et al. 1990].

The genome-wide linkage scans have identified Multiple loci linked to disease and revealed overlap with psoriasis susceptibility loci on chromosomes 1q21 (PSORS4), 3q21 (PSORS5), 17q25 (PSORS2) and 20p12.a strong HLA-C association is described (PSORS1). The peak of linkage of Atopic Dermatitis and psoriasis on chromosome 1q21 overlies the human epidermal differentiation complex (EDC) that spans a region of ~2 Mb [Mischke, et al. 1996]. The genes of the EDC are expressed late during maturation of epidermal cells [Hardas, et al. 1996]. The causes of psoriasis are unknown to date.

There are many pathways resulting up or downregulated in these diseases. Several studies have demonstrated that VEGF expression is increased in lesional psoriatic skin, that the serum levels of circulating VEGF protein are significantly elevated in patients with severe disease, and that VEGF serum levels were directly correlated with disease activity [Bhushan, et al. 1999, Creamer, et al. 1996, Hardas, et al. 1996, Nielsen, et al. 2002]. A major role of VEGF in the pathogenesis of psoriasis was further corroborated by the phenotype of transgenic mice with epidermis-specific overexpression of VEGF. VEGF transgenic mice show enhanced skin vascularity and vascular permeability [Detmar, et al. 1994]. At about 6 months of age, these mice spontaneously develop chronic inflammatory skin lesions that histologically closely resemble human psoriasis [Xia, et al. 2003]. To monitor angiogenesis in vivo, it was created a transgenic mouse, *Vegfr2-luc*, in which a luciferase reporter is under the control of murine *VEGFR2* promoter and allowing to follow the *Vegfr2* promoter switch on by in vivo bioluminescence [Zhang, et al. 2004].

The Wnt signaling pathway, was found to strongly up-regulate VEGF [Zhang, et al. 2001]. There are strong evidence that the Wnt pathway is up-regulated in psoriasis: the expression of *Wnt5a* was 4-fold higher in lesional skin compared to normal skin sample of the same patient [Reischl, et al. 2007]. Moreover there is evidence that some keratinocyte differentiation events are controlled by changes in cell-cell adhesion.  $\beta$ -catenin has a dual function as a component of intercellular adherents junctions and also as a transcription factor as part of the Wnt signaling pathway.  $\beta$ -catenin is not required for keratinocyte proliferation but has been shown to regulate keratinocyte stem cells and hair follicle morphogenesis. In psoriatic skin was observed a strong nuclear localization of  $\beta$ -catenin in suprabasal layers. Moreover was observed a marked decrease in membranous  $\beta$ -catenin and increased cytoplasmic  $\beta$ -catenin at the same level as the nuclear  $\beta$ -catenin. In the uninvolved psoriasis samples there was some nuclear  $\beta$ -catenin but this was much less than in involved psoriasis [Hampton, et al. 2007]. Moreover defects in the Notch pathway are thought to be involved in the pathogenesis of psoriasis. In the skin, *Notch1* has a role in promoting keratinocyte differentiation. In recent years, increasing evidence suggests that Notch may play a key role in linking the control of epidermal proliferation and differentiation. Furthermore, aberrant Notch signaling might contribute directly

to the pathogenesis of skin diseases like psoriasis and Basal Cell Carcinoma. Notch and Delta signaling molecules are up-regulated in the basal and differentiating layers of adult human epidermis, whereas they are under-expressed in disorganised proliferating epidermis. The specific differentiation programme that keratinocytes undergo in skin is under their control. The alteration of the Notch pathway play a role in disrupting the keratinocyte differentiation programme, leading to unorganised suprabasal epidermal strata and dermal invasion [Thelu, et al. 2002]. Recent advances in the study of Notch signaling have confirmed that there is crosstalk between the Notch signaling pathway and a variety of other signaling molecules Wnt  $\beta$ -catenin and the p53 family member, p63. The absence of Notch activity allows Wnt signaling to persist in a tissue where they are normally repressed [Nicolas, et al. 2003]. In addition, Notch counteracts the action of p63 to maintain immature cell characteristics.

## **2.AIM OF THE STUDY**

To better understand the biological function of Prune *in vivo* system, during my Ph.D. work I focused my research interest in the attempt to characterize the transgenic mice overexpressing h-Prune into skin. The purpose of our study was to understand the role of h-Prune in epidermal proliferation and keratinocyte-differentiation using a transgenic mouse model, already present in the laboratory of professor Zollo, in which the transgenic protein expression was driven by a modified Loricrin promoter (ML) obtaining a specific epidermal increase of the protein of interest [Waikel, et al. 1999].

First we wanted to characterize the phenotypic changes carried by h-Prune overexpression in the mouse epidermis.

H-Prune protein is known to date to stimulate cellular motility and metastasis process in a breast cancer model [D'Angelo, et al. 2004]. The role of h-Prune in the increased cell motility can be explained in part to h-Prune interaction with GSK-3 $\beta$  with which cooperatively works to regulate the disassembly of focal adhesions and promote cell migration [Kobayashi, et al. 2006]. GSK-3 $\beta$  covers a fundamental role like key effector of the canonical Wnt pathway (Wnt/ $\beta$ -catenin signaling). Wnt signaling deregulation leads to an imbalance of proliferation and differentiation program [Reya, et al. 2001]

Based on these assumptions we wanted to investigate the effect of h-Prune on GSK-3 $\beta$  downstream regulated genes (canonical Wnt pathways related genes). Due to GSK-3 $\beta$  and h-Prune promote cell motility, we expected that the resulting phenotype of our transgenic mice was related to an hyperproliferation and/or would lead to the defect in keratynocytes differentiation program.

Since h-Prune in combination with GSK-3 $\beta$  increased the cell motility, we wanted to analyze h-Prune expression levels in an hyperproliferative/inflammatory skin disease like Psoriasis. In such disease infact the basal keratynocytes increase their motility migrating to the outer layer of epidermis.

Finally we want to find similarities between the molecular mechanisms activate by h-Prune overrepression in the skin, in the ML-h-Prune transgenic mice and the known defects of signaling pathways which are normally deregulated in the human psoriasis, a multigenetic disorder which show both hyperproliferation, migration and a pro-inflammatory phenotypes.

### **3.MATERIALS AND METHODS**

### **3.1.ML-h-Prune transgenic mice genotyping**

PCR amplification was used to genotype mice by detection of sequences not normally present in the murine genome. The amplified segment of DNA, was detected by agarose gel electrophoresis. The genomic DNA was prepared by digesting mouse tails with 1.5 µg/ml proteinase K (Promega) in 200 µl DNA extraction buffer containing 50 mM Tris pH 8; 100 mM EDTA; 100 mM NaCl; 1% SDS, overnight at 55 °C in a termoblock. The genomic DNA was extracted with phenol/chloroform solution and precipitated with isopropyl alcohol. DNA was resuspended in 50 µl TE buffer (Invitrogen) and stored at +4 °C. The PCR reaction was performed using 2 µl (100 ng/µl) DNA in 50 µl of reaction buffer containing 20 µl of 2.5x Master Mix (5Prime Eppendorf) and 400 nM each primers (sense and antisense). Following primers were used: M-Loricrin forward: 5'-AGG AAT GGA ACA GTC CAG C-3', H-Prune reverse: 5'-GCT CGA GTC TGG GGT GAT CT-3', which amplify a fragment of about 500 bp. The samples for PCR amplification were prepared using a laminar flow hood to avoid contamination. The reaction was carried out as follows: initial denaturation of 5 min at 94 °C and 35 cycles of amplification. Each cycle consists of a denaturation step of 30 sec at 94 °C, an annealing of 45 sec at 55 °C, and extension of 1 min at 72 °C; the extension was prolonged for 10 min during the last cycle.

### **3.2.Protein extraction and western blot analysis**

The cells were washed with PBS and lysed directly on ice for 30 minute with a RIPA buffer containing 1% NP40, 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1x protease inhibitors (Roche Applied Science) and sodium orthovanadate (SIGMA). For lysates derived from skin, tissue were removed from 5-days old mice. Dermal epidermal separation was obtained dipping the removed skin in PBS1x at 60°C for 1', followed to a rapid passage in PBS1x placed in ice. The epidermis was finally slowly pulled from the dermis using a tweezer. The samples were homogenized by using TissueLyser (QIAGEN) at a frequency of 30 Hz for 1 minute. To allow the homogenization of tissue, beads were inserted into the 2 mL tube containing the sample and RIPA buffer. To detect the relative levels of protein quota we used the following antibodies: h-Prune monoclonal antibody (able to recognize both human and murine Prune protein), anti-β-catenin and anti-Filaggrine (BD Biosystems), anti-activated-β-catenin (Millipore), anti-β-actin (Sigma), Hes 1 and Cyclin D1 (Santacruz Biotechnology), p21, Cytokeratin 15, Cytokeratin 14, Cytokeratin 16, NICD1, total GSK-β, Ser9-P-GSK3β and Hey1 (abcam) and horse-radish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare Life Sciences, Milan,Italy).

### **3.3.Histological and Immunohistochemical analysis.**

Skin samples from transgenic littermate mice were fixed in PBS-buffered 4% paraformaldehyde for 16 hours and then dehydrated before embedding into paraffin. Embedded skin samples were sectioned (7µm) and deparaffinized in Bio-Clear (Bio Optica) for 15 minutes. Samples were rehydrated by using a scalar concentration of ethyl alcohol (100%, 90% and 70% of ethyl alcohol) and were stained with haematoxylin and eosin (H&E) or analyzed by immunohistochemistry. Haematoxylin and eosin staining, skin samples after rehydration were washed in distilled water and staining in Harris haematoxylin solution for 8 minutes. After washing in running tap water for 5 minutes, samples were differentiated in 1% acid alcohol for 30 seconds and washed again in running tap water for 1 minute. Skins were washed with a 0.2% ammonia solution for 30 seconds to 1 minute and, after it have been washed with running tap water for 5 minutes, were rinsed in 95% ethyl alcohol 10 dips. Counterstain was made in eosin-phloxine solution for 30 seconds to 1 minute. After dehydration through 95% ethyl alcohol and 2 changes of ethyl absolute alcohol, from 5 minutes each, skin samples were cleared in Bio-Clear (Bio Optica ) and mounted with Eukitt (Bio Optica). Immunohistochemical analysis was performed using Vectastain Elite ABC Kit (Vector Laboratories Inc.) according to the manufacturer's instructions. Sections were pre-treated in a microwave oven for 5 minutes at high energy and for 15 minutes at low energy in 10 mM Na Citrate (pH 6.0). The prune monoclonal antibody (Anti-Prune) was used at dilution 1:1000 for mouse tissues and 1:500 for human skin. Colorimetric reactions were carried out using DAB (Vector, Vector Laboratories Inc.) according to the manufacturer's instructions.

### **3.4.Measurement of epidermal thickness**

The quantification of the interfollicular epidermal thickness of ML-h-Prune mice was performed by measuring the distance between the basal layer and granular layer. In each experiment were analyzed three different mice per genotype, measuring ten regions per mouse.

### **3.5.Skin permeability assay.**

This assay is based on skin barrier-dependent penetration by histological dyes. The basis of this skin modification is unknown but is likely to involve extraction of polar lipid [Wertz and Downing 1987]. Unfixed, untreated embryos were incubated for 1-5 minutes (45 minutes for 6 days old mice) in methanol and rinsed in PBS, followed by incubation in 0.1% toluidine blue. After staining, embryos were photographed using a Zeis Stemi SV11 microscope with transmitted and surface illumination. For the 6-days old mice photographs were taken with a simple camera placing the mice on a white background. Scanned images were processed with Adobe Photoshop.

### 3.6. Cell culture and transfection

HaCat cells, a spontaneously immortalized human epidermal keratinocyte cell line grown at 37°C under 5% CO<sub>2</sub> in Dulbecco's modified Eagles' medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. HaCat cells were transiently transfected with plasmid by lipofection using TransFectin Lipid reagent (BioRad) according to the manufacturer's instructions. Cells were replated 24 h before transfection at an optimal cell density in 10 ml of culture medium in 100 mm tissue culture dishes. For transfection, 40 µl of TransFectin Lipid reagent were incubated with 15 µg of indicated plasmids, in 3 ml of serum free DMEM for 30 min at room temperature. Cells were transfected by changing the medium with 1 ml of DMEM containing the plasmids and LipoFectamine lipid reagent, followed by incubation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 48 h. To have a confirmation of transfection, in each transfection mix, together with the plasmid transfected, 700 ng of pEGFP vector was cotransfected with the indicated plasmid. After 48 h of incubation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> the GFP was detected by a fluorescence microscope.

### 3.7. Tissue RNA extraction.

The animals were sacrificed by CO<sub>2</sub> saturated atmosphere. Immediately after death a small piece of skin (≈ 2cm) was taken from the back of mice using a little scissor, and epidermis was separated from dermis as previously described. Having obtained the mice genotype the epidermis were thawed in ice and the tissues were lysed by TissueLyser (QIAGEN), placing the single epidermis in 2ml-tube in 1ml of trizol, at a frequency of 30 Hz for 1 minute. The lysate was clarified by centrifugation at 13000 g for 10 min. Supernatant was transferred to a clean tube and the total RNA were extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was calculated using a NanoDrop spectrophotometer at 260 nm absorbance. 3 µg of total extracted RNA was reverse transcribed with Iscript kit (BioRad). The cDNA was diluted twenty-fold prior to PCR amplification. Notch targets, Wnt targets and Prune levels mRNA expression were evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR). Reactions were performed in a 10 µl volume with 0,6 µM primers, 45 ng of cDNA template and SYBR mix (Applied Biosystems) that includes Taq DNA-pol and dNTPs. Negative controls included PCR reactions with cDNA omitted. Each amplification reaction was done in duplicate. To confirm amplification specificity, PCR products from each primer pair were subjected to melting curve analysis. Data were analyzed with  $\Delta\Delta C_t$  method. The results are presented as normalized ratio of the expression of specific molecule mRNA to  $\beta$ -Actin. The oligo sequence utilized for cDNA amplification are as follow:

**C-MYCforw:** *CTCCTCGAGCTGTTTGAAGG*  
**C-MYCrev:** *AGCAGCTCGAATTTCTTCCA*  
**HES1forw:** *TCATCCCGGTCTACACCAGC;*  
**HES1rev:** *GGAGCCACTGGAAGGTGACA*  
**ASCL1forw:** *CCTGGGAATGGACTTTGGAAG*

**ASCL1***rev: TGACGTCGTTGGCGAGAAA*  
**mJAG1***\_for: GGTGGAGGCCTGGGATTC*  
**mJAG1***\_rev: TGTTTCAGTGTCTGCCATTGC*  
**mBirc5***\_for: TGTTTTTCTGCTTTAAGGAATTGG*  
**mBirc5***\_rev: CATCTGCTTCTTGACAGTGAGGAA*  
**mHES5***\_forw: ATGCTCAGTCCCAAGGAGAAAA*  
**mHES5***\_rev: GCTCCAGCAGCAGCTTCAG*  
**mCycliD1***\_for: TCTTACTTCAAGTGCGTGCAGAA*  
**mCycliD1***\_rev: AGCGGGAAGACCTCCTCTTC*  
**mp27***\_for: ACAATCCGGCTGGGTTAGC*  
**mp27***\_rev: AACCGTCTGAAACATTTTCTTCTGT*  
**mK15***\_for: AGAGCCCAGCCAGTCCAGAT*  
**mK15***\_rew: AACGCGCGAGTTGTCAATG.*

### **3.8. Animal experiment.**

In vivo imaging was performed as previously described [Zhang, et al. 2004] using IVIS imaging system (Xenogen). 5-days old pups *Vegfr2-luc/ML-Prune* transgenic mice, were anesthetized with isoflurane and injected intraperitoneally with 150 mg/kg luciferin (D-Luciferin Firefly, potassium salt, Caliper Life Science). Ten minutes after luciferin injection, mice were imaged for 1 to 5 minutes. To quantify the bioluminescence the integrated fluxes of photons (phot. per sec) within an each area of interest were determined using LivingImage 3.2 software (Xenogen). The data were analyzed using Excel software or the QuickCalc software.

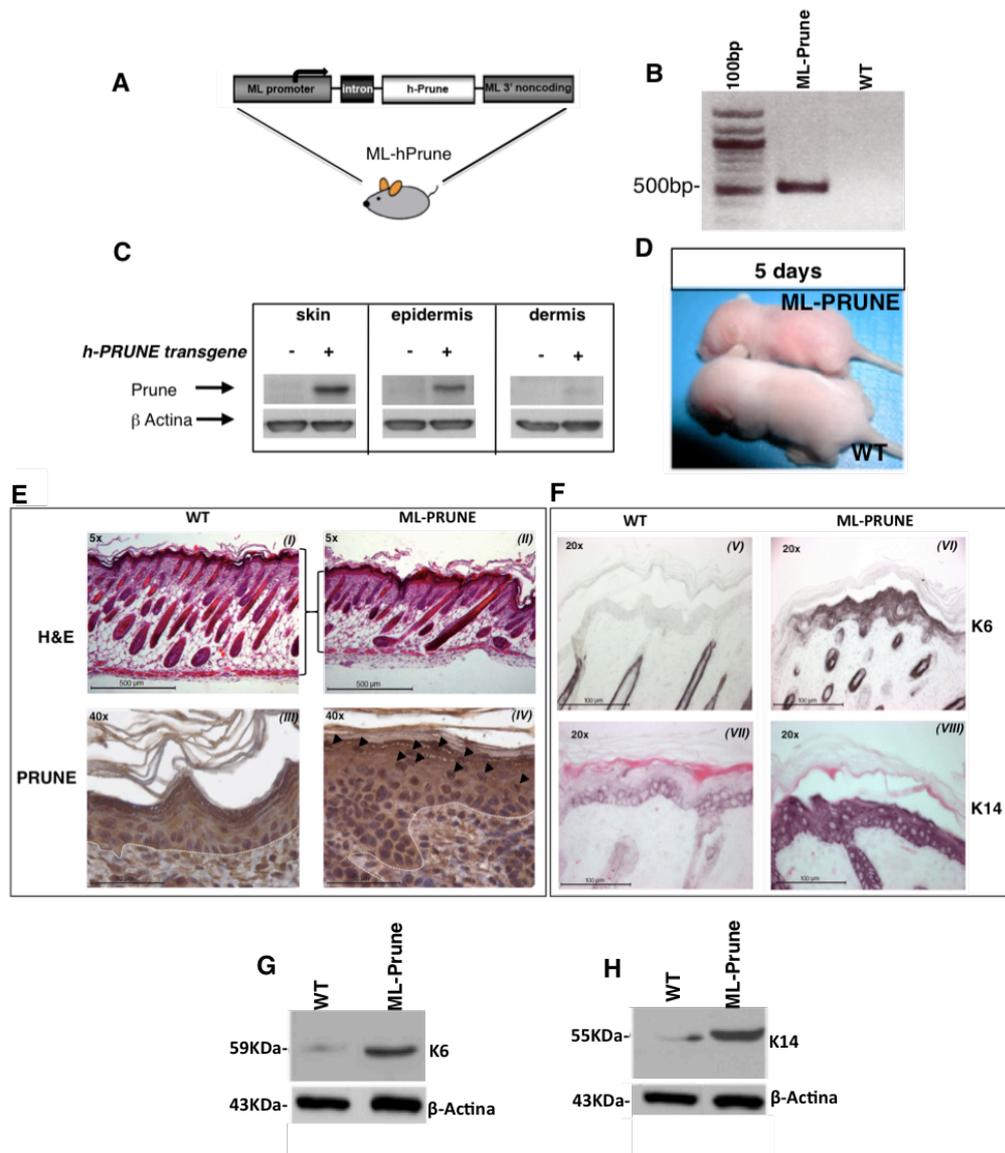
### **3.9. Luciferase reporter assays and TOP/FOP assay**

Luciferase reporter assays were performed using the Dual Luciferase<sup>®</sup> Reporter Assay (Promega). HaCat cells and HEK293 cells were transfected with the PolyFect transfection Reagent (Qiagen) according to the manufacturer's instructions. In brief,  $2 \cdot 10^5$  cells/well were seeded in a 6-well plate the day before transfection. 500 ng of luciferase reporter plasmid and 100 ng of pRL-CMV (Promega) were transfected. Cells after 48 hours of transfection with indicated vectors, were lysed with Passive Lysis Buffer (Promega), and Luciferase activity present in cellular lysates was assayed following the manufacturer's instructions. Light emission was quantified with the luminometer LUMINO (Stratatec). Relative Luciferase Units (RLU) were calculated. For the TOP/FOP assay we used the same procedure first described. The TK-TOP and TK-FOP optimal and mutated TCF luciferase-reporter constructs have been described previously [Staal, et al. 1999].

## **4.RESULTS AND DISCUSSION**

#### 4.1. Phenotypic characterization of ML-Prune transgenic mice

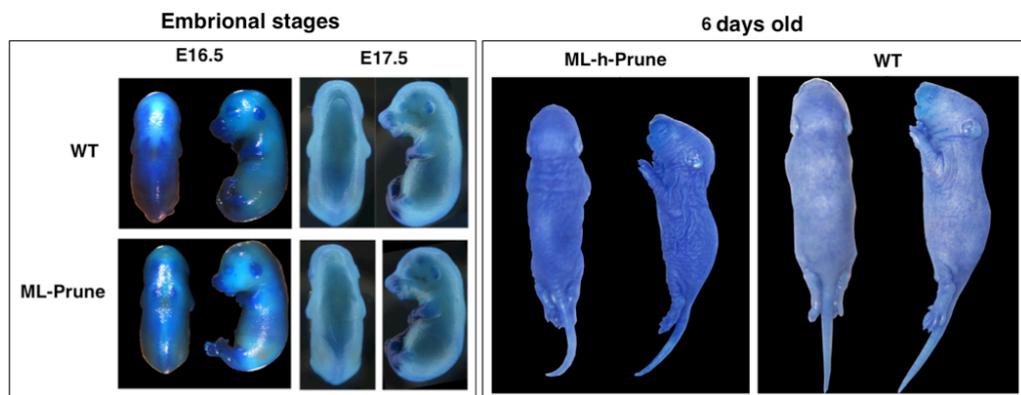
The transgenic mice ML-h-Prune were identified by genotyping (fig. 6 B). The expression of h-Prune were detected by a W.B. analysis first of the total skin, and after separately epidermis and dermis protein extracts using an anti-Prune antibody, able to detect both human and mouse proteins (fig 6 C). The result of WB shows an epidermal specific expression of h-Prune (fig 6 A). At birth transgenic pups were indistinguishable from their littermates, after 4-5 days transgenic mice (TG) were smaller than wild type littermates. ML-h-Prune transgenic mice showed a significantly thinner and more wrinkly skin compared to WT littermate (fig 6 D), which appeared red and flaky, showing an inflammatory like phenotype. Histological analysis of skin architecture on the dorsal skin, examined using haematoxylin and eosin-stained sections, revealed that the epidermis of 5-old days ML-Prune transgenic pups appeared moderately hyperplastic, with a thinner dermis and higher granular and spinous layer than WT littermate (fig 6 E, panel *I* and *II*). Moreover immunohistochemical analysis of skin section with an anti-Prune, confirmed the overexpression of the protein seen for W.B. and showed an higher number of nucleated cells in the upper layer of ML-h-Prune epidermis respect to the WT (fig 6 E, panel *III* and *IV*). We think this is due to a failure of cell differentiation program leading the immature cells to migrate to the outer layers of epidermis. The hypothesis about the alteration of keratinocytes differentiation was strengthened by the immunostaining of skin section with an antibody direct against keratin 6 (K6): K6 regularly used as marker of proliferating cells in hair follicles and in skin pathologies [Weiss, et al. 1984] were found confined to the companion layer of the hair follicles in wild type controls while in transgenic mice it was found aberrantly expressed also in the epidermis (fig 6 F, panel *V* and *VI*). Moreover keratin 14, marker for basal proliferating cells [Fuchs and Green 1980], analyzed by immunohistochemical staining showed a markedly aberrant immunoreactivity in superbasal layers of epidermis in transgenic mice (fig 6 F, panel *VII* and *VIII*). The overexpression of both cytokeratins in the ML-h-Prune transgenic skin was confirmed by WB analysis (fig 6 G and fig 6 H). The transgenic phenotype gradually disappeared and it was absent in adult animals, although the transgene was still expressed at moderated levels in adult mice. This phenomenon can be explain by the loricrin used promoter level of expression, highly functioning in the first weeks of mice inborn life and to the tight level of regulation occurring in epidermis that balances the overexpression of h-Prune, causing hyperproliferation, with concurring altered differentiation and apoptosis. Although this phenomenon is common in epidermal transgenic models [Waikel, et al. 1999] these results will be further investigated in the near future. The higher expression of the two keratins in the transgenic mouse skin was confirmed by WB (fig 6 C).



**Fig 6. ML-h-Prune transgenic mice characterization.** (A) The transgenic construct to obtain ML-h-Prune transgenic mice was generated by inserting the human h-Prune cDNA into the 6.5 Kb mouse modified loricrin expression vector. (B) PCR analysis to detect the presence of transgene in mouse epidermis. (C) W.B. analysis of ML-h-Prune transgenic back-skin, showed that the protein was expressed in high levels specifically into epidermis. (D) At 5 days of life transgenic mice were smaller than wild type littermates and showed a more wrinkly skin with an epidermis moderately hyperplastic, with a thinner dermis and higher granular and spinous layer (E-I and E-II). Immunohistochemical analysis of skin section with an anti-Prune, confirmed the overexpression of the protein seen for W.B. and showed an high number of nucleate cells in the upper layer of ML-h-Prune epidermis respect to the WT (E-III and E-IV) (the dashed white line demarcates the separation between epidermis and dermis). (F) Immunohistochemical analysis with K6 and K14 antibody showed an higher and ectopic expression of both proteins in the ML-h-Prune transgenic mice (F-V and F-VI; F-VII and F-VIII). The higher expression of K6 and K14 were confirmed by WB analysis (G and H)

#### 4.2. Deregulation of skin barrier in ML-h-Prune transgenic mice

Many mouse models carrying alterations to the skin structure show an alteration also of the skin barrier [Koch, et al. 2000, Matsuki, et al. 1998]. The formation of a functional epidermal barrier occurs between E16.5 and E17.5 days and coincides with the formation of the stratum corneum [Hardman, et al. 1998]. To investigate if the skin barrier of ML-h-Prune transgenic mice was functionally, we performed an assay of skin permeability with Toluidine Blue staining. In these experiment the mouse analyzed belonged to the same progeny, so they were analyzed at the same developmental stage. In the figure 7 (left panel) we can observe that during embryonal stages (E16.5 and E17.5) of formation of skin barrier, no differences occur between ML-h-Prune transgenic mice and the WT littermate. Between the 5<sup>th</sup> and 6<sup>th</sup> day of life, two days after the maximum expression peak of transgene, the ML-h-Prune model showed an higher absorption of the dye respect to the WT mouse, reflecting an higher permeability of the skin (fig 7 right panel). These results lead us to think that h-Prune is able to interfere in the normal process of skin homeostasis. We therefore decided to investigate the molecular pathways underlying the phenotypical alteration observed in the h-Prune transgenic skin.

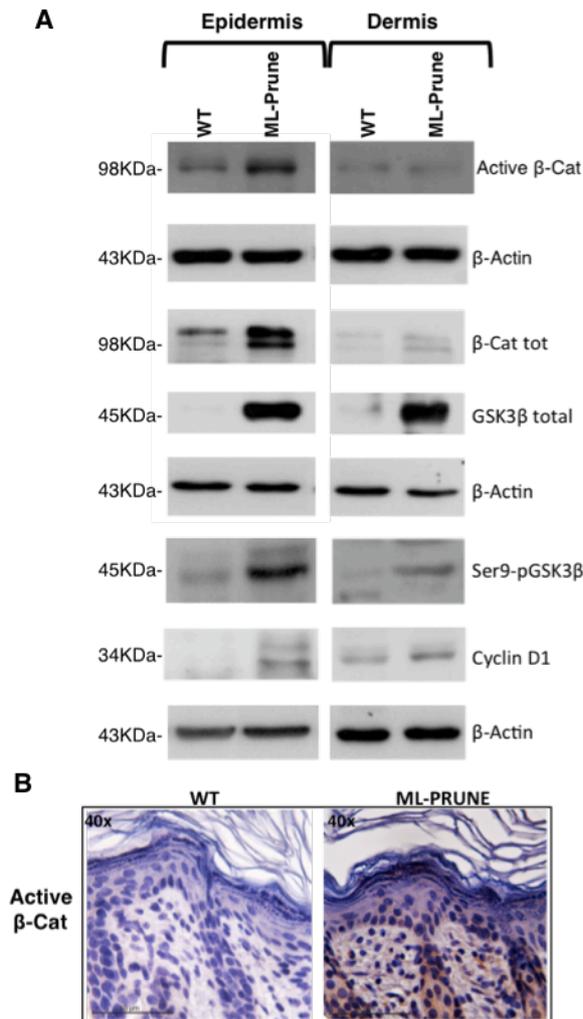


**Fig 7. Skin barrier alteration of the ML-h-Prune transgenic mice.** No differences in skin barrier formation occur between ML-h-Prune transgenic mice and the WT littermate during embryonal stages (E16.5 and E17.5) (left panel). The ML-h-Prune model showed an higher absorption of the dye between the 5<sup>th</sup> and 6<sup>th</sup> day of life, two days after the maximum expression peak of transgene (right panel), thus indicating that the skin barrier at this stage is fully retaining the blue dye in the ML-h-Prune animals compared to wild-type animals.

### 4.3. Altered Wnt signaling in ML-h-Prune transgenic mice.

From literature h-Prune and GSK-3 $\beta$  are known to interact. In vitro binding studies using recombinant proteins demonstrated that GSK-3 $\beta$  bound directly to h-Prune [Kobayashi, et al. 2006]. In addition, recent studies have found that GSK-3 $\beta$  is a central regulator of cell polarity and cytoskeleton dynamics [Nusse 2008]. In vivo, GSK-3 $\beta$  activity is inhibited by Wnt signaling, which in turn leads to  $\beta$ -catenin stabilization, which plays a critical and near universal role in epidermal stem cell (SC) activation and migration [Fuchs 2009, Nusse 2008].

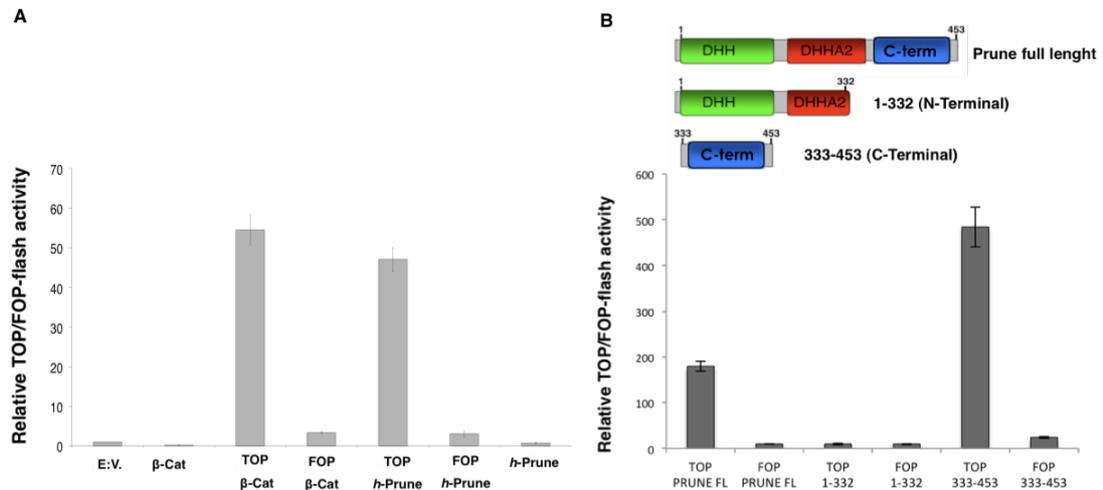
To investigate whether h-Prune was able to mediate a Wnt pathway activation, we examined epidermal active  $\beta$ -catenin performing blot analysis on both epidermis and dermis protein lysates, isolated from 5 days old ML-h-Prune mice. We observed a substantial increase in the active  $\beta$ -catenin protein amount (fig 8 A left column), confirmed also by immunohistochemical that showed a markedly aberrant nuclear staining of the protein (fig 8 B). This phenomenon was not observed in WT mice. These results prompted us to evaluate the activity of GSK-3 $\beta$ . The ability of GSK-3 $\beta$  to target substrates for degradation is attenuated through phosphorylation of the serine-9 residue [Doble and Woodgett 2003]. We demonstrated using specific antibodies specific for pSer9-GSK3 $\beta$ , that h-Prune overexpression enhances levels of the inactive form of GSK3 $\beta$  (pSer9-GSK3 $\beta$ ) in the epidermis of these mouse model, observing that the proportion of the inactive GSK-3 $\beta$  protein was increased in ML-h-Prune mice (fig 8 A left column). Of note we observed also that GSK-3 $\beta$  protein level was found increased. It has been shown that cyclin D1 acts as direct target gene for  $\beta$ -catenin [Shtutman, et al. 1999], in agreement with this finding, western blot analysis showed that Cyclin D1 protein was found indeed activated in the epidermis of ML-h-Prune mice [Zhang, et al. 2011].



**Fig 8. Levels of canonical Wnt pathway related protein in epidermis and dermis of ML-h-Prune transgenic mice.** (A) Western blot analysis of separated proteins extracts of epidermis and dermis of ML-h-Prune transgenic mouse compared to WT mouse. The transgenic mice showed higher inactive pSer9-GSK3β and consequent active-β-catenin levels in the epidermis. GSK3β levels showed no variation, while high levels of total β-catenin was found in the epidermis of transgenic animals. Also Cyclin D1 levels were increased in the epidermis of ML-h-Prune transgenic mice. (B) IHC analysis of ML-h-Prune transgenic skin showed high nuclear-β-catenin staining respect to WT mice.

As we have previously shown, h-Prune is a positive regulator of β-catenin signaling in our mouse model, for this reason we examined whether in *in-vitro* experiments h-Prune transfection drives β-catenin up-regulation in HACAT cells. To quantify β-catenin/LEF-mediated transcriptional activation, we transfected HACAT keratinocytes with the TOP-flash reporter containing the LEF binding motifs and measured luciferase activities [Korinek, et al. 1997]. FOP-flash vector with mutated LEF sites were used as control. As shown in figure 9 A, the luciferase activity were significantly higher in h-Prune transfected keratinocytes compared with empty

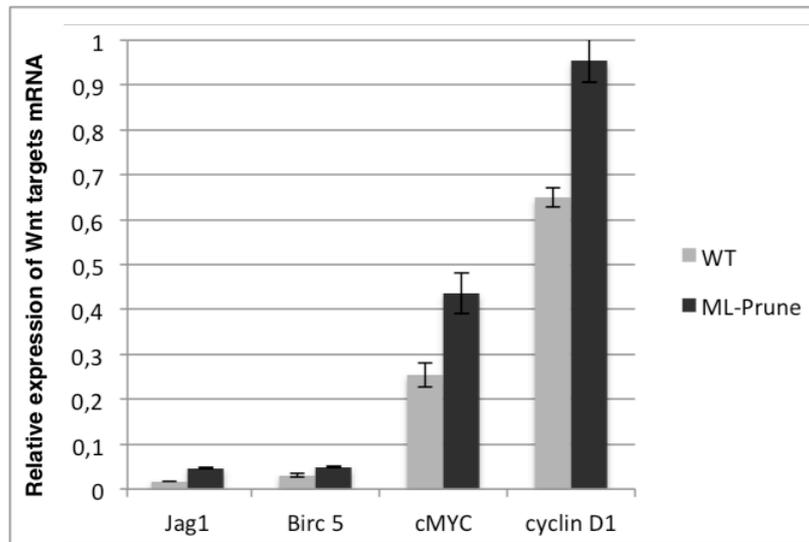
vector transfected cells. The h-Prune protein comprises various domains; Kobayashi et al. in the 2006, showed that amino acid region 333 to 453 of h-Prune was necessary and sufficient for the complex formation with GSK-3 $\beta$ . Here we examined, using the same methodology, which region was able to activate  $\beta$ -catenin/LEF-mediated transcriptional activation. Figure 9 B shows that h-Prune full length (amino acids 1-453) and h-Prune C-Terminus (amino acids 333-453) significantly activated transcription of reporter gene while h-prune N-Terminus (amino acids 1-332) is not able to perform this at similar strength.



**Fig 9. Increased ignition of Tcf/Lef mediated transcription upon h-Prune overexpression.** (A) HACAT keratinocytes co-transfected with h-Prune expression vector and TOP-flash reporter showed increased levels of luciferase signal compared to empty vector/ TOP-flash reporter transfected cells. FOP-flash vector with mutated LEF sites was used as control. (B) Prune C-terminal region transfected cells showed higher luciferase reporter signal compared to Prune full length transfected cells and to Prune N-terminal transfected cells, suggesting that the effective domain able to impair the Wnt pathway is the amino acid sequence 333-453 (C-terminal region).

#### 4.4.H-Prune increases the transcription of Wnt target genes in the transgenic mouse epidermis

Since h-Prune was able to increase  $\beta$ -Catenin transcriptional activity, we additionally explored whether or not h-Prune regulates the canonical WNT target genes transcription. If h-Prune overexpression enhance WNT signaling throughout pSer9-GSK3 $\beta$  stabilization and consequently by inhibition of  $\beta$ -catenin degradation we could gain additional indirect evidence through the enhancing of known early WNT target genes. For this purpose, we monitored the mRNA levels of JAG1, Survivin (Birc5 gene transcript), cMyc, and cyclin D1 by real-time PCR using RNA from TG and WT epidermis. Within this assay, as expected, we found that JAG1, Survivin (Birc5 gene transcript), cMyc, and cyclin D1, showed enhancement of ~2,6 - 1,6 - 1,7 and 1,4 fold respectively (fig 10).



**Fig 10. Increased mRNA rates of Wnt target genes in the ML-h-Prune transgenic epidermis.** Real time PCR analysis detected higher mRNA levels of Wnt related genes in the transgenic epidermis: JAG1, Survivin, cMyc, and cyclin D1 was all upregulated in the ML-h-Prune transgenic mouse compared to WT mouse.

For this purpose the next step of our work was to study the Notch pathway activation in ML-h-Prune transgenic epidermis.

#### **4.5. Negative regulation of Notch signaling in ML-h-Prune transgenic skin and phenotypic alterations.**

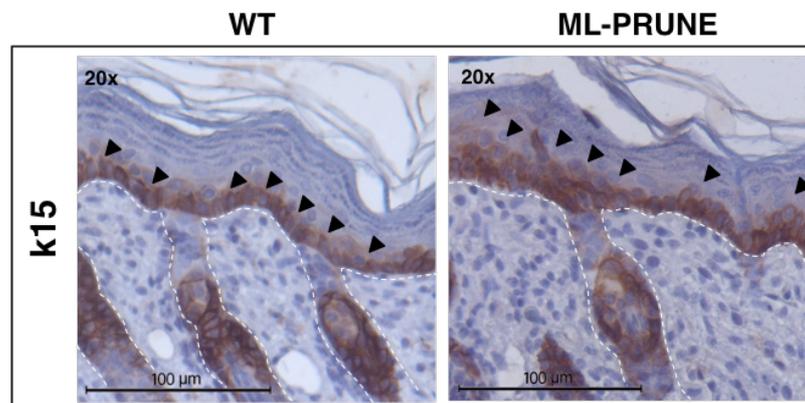
We have previously described several skin differentiation defects observed in our transgenic mouse model. Notch signaling has a key role in promoting keratinocyte differentiation exerting a growth-inhibitory function that can be attributed to CBF-1-dependent upregulation p21<sup>cip1</sup> (Cdkn1a) [Lefort and Dotto 2004, Lefort, et al. 2007]. In the embryonic mouse epidermis, Notch1 intracellular domain (NICD1) is ubiquitous in supra-basal keratinocytes selecting the spinous fate [Blanpain, et al. 2006, Lin and Kopan 2003, Pan, et al. 2004]; after birth, NICD1 is detected transiently in a small fraction of spinous cells [Lee, et al. 2007]. This pattern of Notch1 activation is consistent with its proposed role in suppressing proliferation and promoting differentiation through the modulation of several targets [Blanpain, et al. 2006, Mammucari, et al. 2005, Okuyama, et al. 2004, Rangarajan, et al. 2001]. Moreover in vitro studies have shown that following, ligand binding and proteolytic cleavage, intracellular domain of Notch must be phosphorylated by GSK3- $\beta$  to be active and capable to migrate in the nucleus to enhance the transcription of target genes [Espinosa, et al. 2003, Foltz, et al. 2002].

In searching for potential alterations of this pathway, in our transgenic mouse model (which shows clear pro-differentiation defects), we investigated whether Notch signaling might be altered.

Therefore, we tested whether Notch activity is altered upon h-Prune overexpression and whether, h-Prune impairs or enhances Notch signaling.

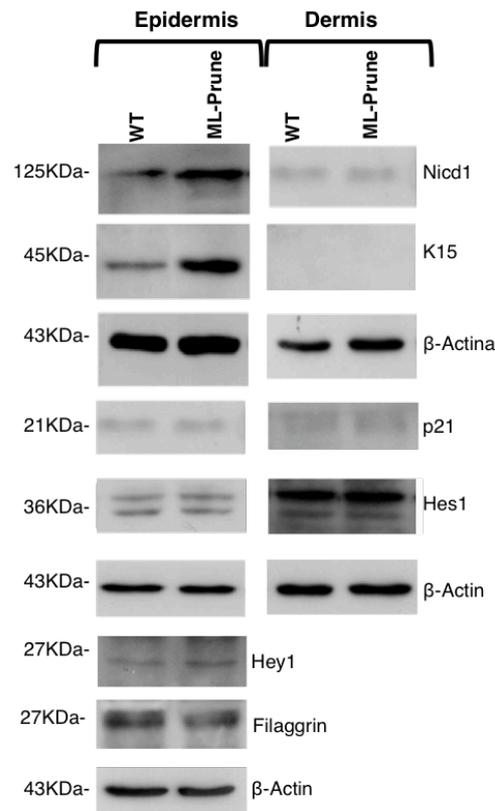
To determine whether h-Prune was able to alter Notch signaling we first measured by western blot experiments, the amount of cleaved intracellular domain of Notch (NICD, activated form of Notch) that was present. We found that, in epidermis of ML-h-Prune TG mice compared with WT littermate, the amount of NICD was markedly increased (fig 12). Next we explored the protein expression of Notch target genes such as p21, hes1 and hey1 [Lefort and Dotto 2004]. By contrast western blot analysis from these mice revealed no variation in protein amount. The lack of an overt variation, following NICD activation, of these targets suggest that h-Prune/GSK3- $\beta$  interaction also influences Notch signaling activation in the skin. Of note filaggrin, that is required for keratin bundle formation and correct biogenesis of the stratum corneum, showed a decreased level of staining in ML-h-Prune mice, confirming an impaired differentiation program and raising the possibility that Notch signaling may be altered in these mice.

Notch signaling induces suprabasal-specific genes such as Keratin1 and Keratin10 and represses basal genes such as Keratin15 (K15) and the  $\beta$ 1 and  $\alpha$ 6 integrins [Moriyama, et al. 2008]. Immunohistochemical analysis of keratin 15 in ML-Prune TG skin showed a normal distribution of K15 both in WT and ML-h-Prune mice. However in ML-Prune mice were observed a double layer of K15 positive cells in the basal compartment of epidermis compared to WT littermate (fig 11). No substantial histological alterations were found in the bulge region, dermal papilla and infundibulum.



**Fig 11.** K15 staining of ML-H-Prune transgenic skin showed a bilayer of K15 positive cells (arrowheads) respect to the single layer of K15 positive basal cells in the WT mice.

Western blot analysis on protein skin extracts confirmed that K15 was expressed at higher levels in the ML-h-prune TG mice and that their up-regulation is correlated to h-Prune levels of expression (fig 12).

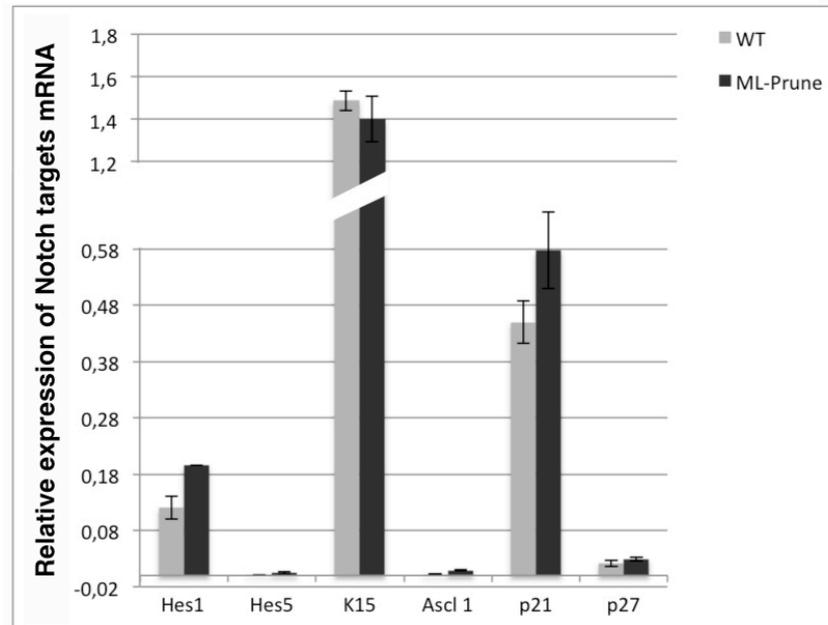


**Fig 12. Increased levels of Notch1 receptor cleavage in the ML-h-Prune transgenic skin.** WB analysis showed an high cleavated an internalized Notch1 membrane receptor (NICD1). This cleavage however did not lead to an increase of Nocht1 transcriptional target as Hes1 and Hey 1 and related protein as p21 in ML-h-Prune transgenic skin. Instead the inversely correlated with Notch pathway activation cytokeatin 15 (K15) showed a clear increase of its expression in the transgenic epidermis.

#### 4.6. The higher rate of Notch receptor cleavage is not reflected in the activation of its transcriptional targets in the ML h-Prune skin

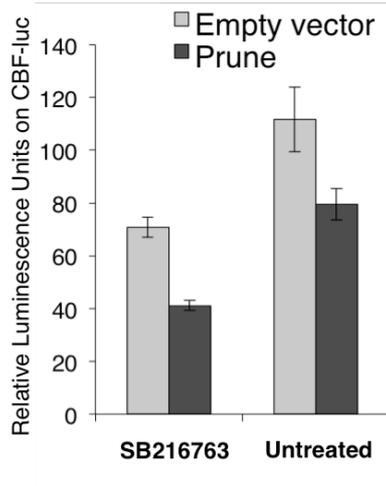
To gain insight into the role of Notch signaling, we further investigated, why we did not observed any protein variation although upstream pathway was found activated. To address this finding we asked whether the impairment might be due to a transcriptional level or a translational level. By qRT-PCR we analyzed the mRNA levels of the canonical Notch targets and genes related to its activation. The best characterized targets of Notch/CBF-1/MamL mediated activation are members of the HES and HERP families of bHLH transcriptional repressors [Iso, et al. 2003]. Furthermore the cyclin/CDK

inhibitors p21<sup>WAF1/Cip1</sup> and p27 have emerged as important targets under positive Notch1 control in mouse keratinocytes, both in culture and in the intact epidermis *in vivo*, which mediate the growth inhibitory effects of Notch1 activation [Nicolas, et al. 2003, Philipp, et al. 1999, Rangarajan, et al. 2001]. mRNA expression of Notch canonical targets Hes 1, Hes 5, p21, and p27 were found invalidated between the two mouse models (fig 13).



**Fig 13. Detection of transcriptional rate of Notch related genes.** The epidermal mRNA amount of direct targets of canonical Notch signaling like Hes 1, Hes 5 as well as Notch related genes like p21, p27 and K15 was not significantly upregulated in ML-h-Prune transgenic epidermis respect to control.

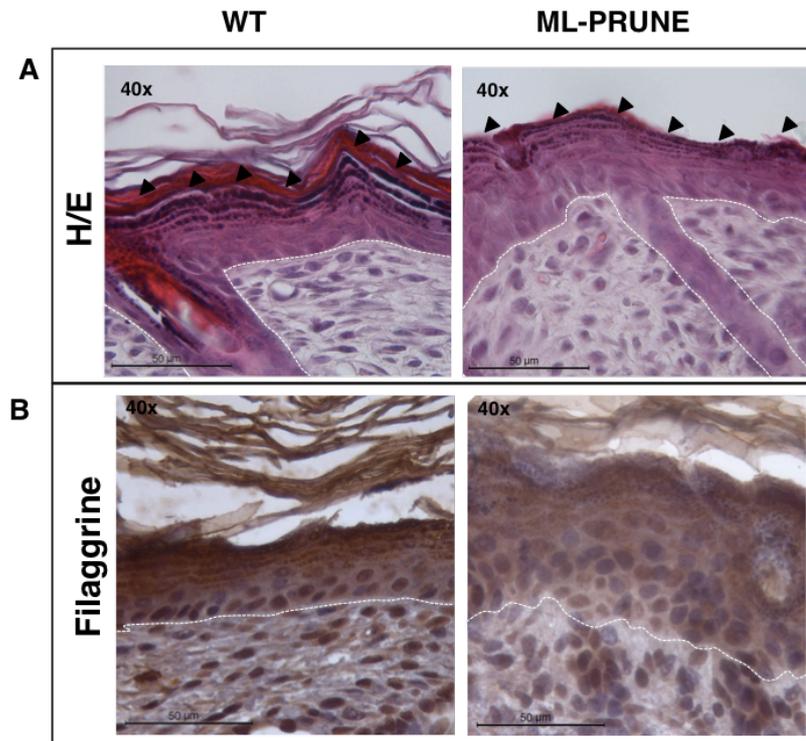
To obtain an umpteenth confirm of transcriptional inhibition exerted by h-Prune on Notch signaling we carried out additional experiment using Notch luciferase reporter vector containing multiple CBF1 binding sites (CBF-luc). For this purpose Hek 293 cells were co-transfected with CBF-luc and h-Prune expression vector (pCS4-3xflag-H-Prune) or CBF-luc and empty vector as control. 24 hours later, transfected cells were plated and treated or not for additional 12 h with a potent GSK3 $\beta$  inhibitor SB216763. Then the cells were lysed and the luciferase was detected. As is shown in figure 14, h-Prune transfection led to an impairment of Notch transcriptional activity, revealed by a decrease in the luciferase amount. Moreover in the SB216763 treated-h-Prune transfected cells we observed further inhibition of the luciferase signal thus indicating that SB216763 and h-Prune cooperatively act on Notch transcriptional impairment. This data strengthens the evidence that Notch signaling impairment occurs at transcriptional level, following GSK3 $\beta$  inactivation carried out by h-Prune.



**Fig 14. Effect of H-Prune overexpression and GSK3 $\beta$  inactivation on the downstream Notch signaling.** Hek 293 cells transfected with a luciferase reporter vector containing multiple CBF1 binding sites (CBF-luc) and with an expression vector for h-Prune protein (pCS4-3xflag-H-Prune) treated and untreated with GSK3 $\beta$  inhibitor SB216763. In the untreated cells Prune transfection carried a reduction of the CBF mediated transcription that was comparable to the empty vector transfected - SB treated cells. A further reduction of the luciferase signal was observed in the h-Prune transfected - SB treated cells.

#### **4.7. Phenotypical alterations in ML-h-Prune transgenic skin related to downregulated Notch pathway.**

The results described above showed an interesting *in-vitro and in-vivo* role for h-Prune in the regulation of Notch signaling, thus indicating that h-Prune can function as suppressor of Notch signaling in the skin. To test this hypothesis, we observed the phenotypical alteration related to notch signaling deregulation. In the stratum corneum the keratin filaments align into macrofibres, under the influence of Filaggrin, the protein component of the keratohyalin granule, responsible for keratin filament aggregation: this fiber with the lipid secreted by keratinocytes are the main component of the skin barrier. Loss of canonical Notch signaling causes severe epidermal barrier and differentiation defects highlighted by reduced spinous, granular, and cornified layer cells [Blanpain, et al. 2006]. Hematoxylin and eosin-stained sections, revealed that the epidermis of 5-old days ML-Prune transgenic pups shows disrupted epidermal macrofibres with less compact clusters of keratohyalin granule (fig 15 A). Moreover immunohistochemical analysis against Filaggrine, to monitoring the distribution of this protein in the epidermal layer, showed a lesser immunoreactivity in the transgenic mice compared with WT control with the signal not located in the stratum corneum but rather spread in the thickness of suprabasal layers (fig 15 B). This data confirm that the basal compartment of transgenic mice is less exposed to Notch signaling which leads to hyperproliferation of the superbasal layer, in agreement with mouse models for Notch KO founded in literature [Lefort, et al. 2007, Moriyama, et al. 2008, Nicolas, et al. 2003].

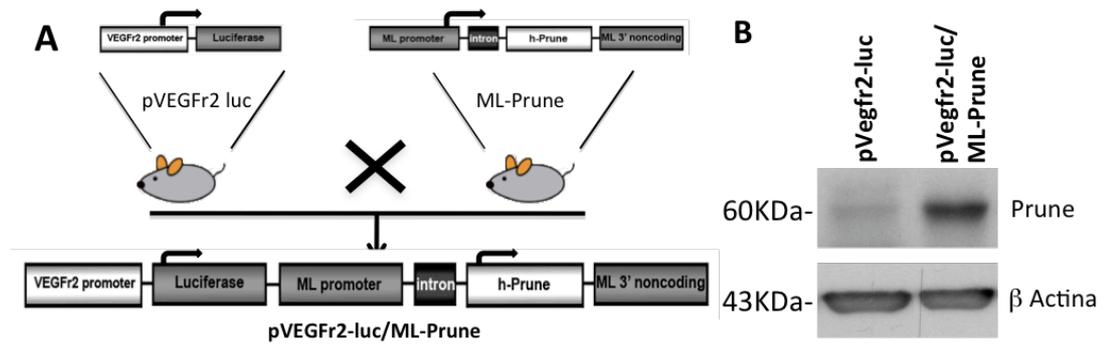


**Fig 15. Phenotypic alteration of ML-H-Prune transgenic epidermis related to Notch pathway decreasing activation. (A)** H/E staining of transgenic epidermis revealed disrupted epidermal macrofibres with less compact clusters of keratohyalin granule into transgenic epidermis. **(B)** IHC against filaggrin showed less marked staining in the transgenic mice compared with WT control. The signal was not located in the stratum corneum but rather spread in the thickness of suprabasal layers.

#### 4.8. Following *in vivo* the Wnt activation. The ML-h-Prune/pVegfr2-luc transgenic mice.

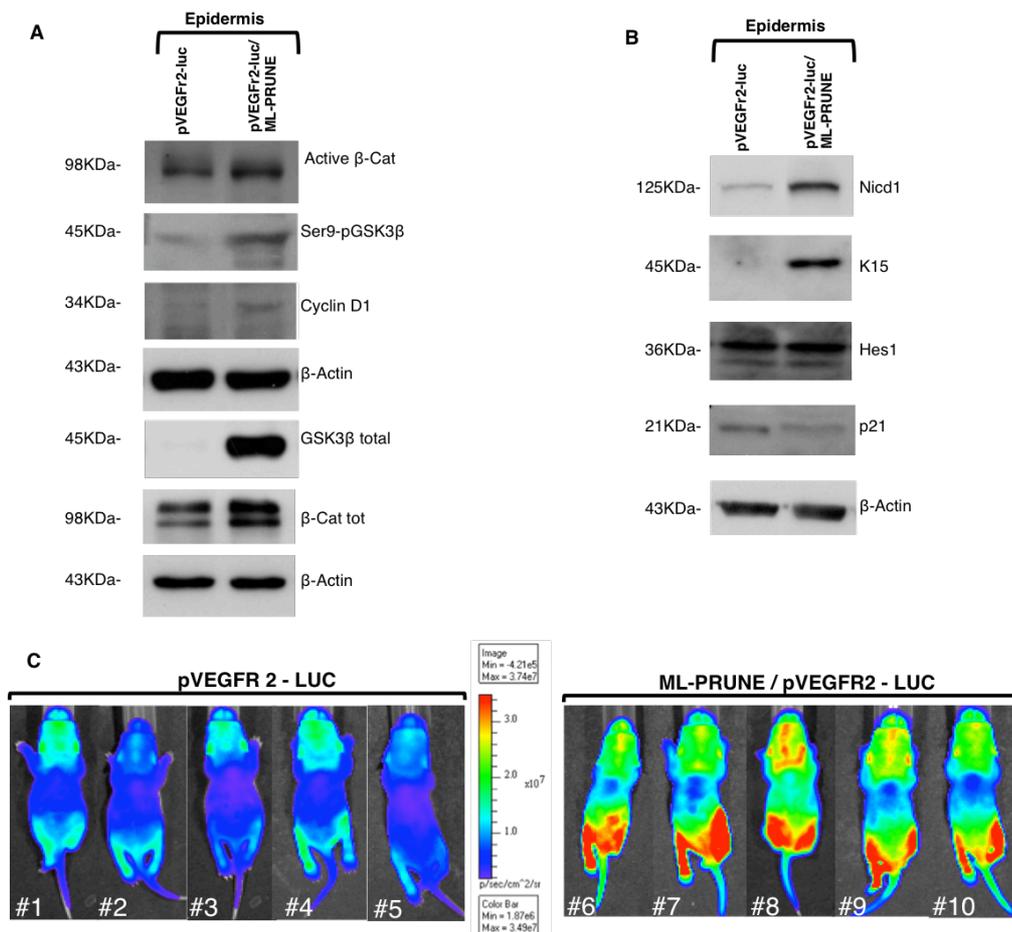
The aim here was to confirm the above-described evidence concerning the *in vivo* effect of h-Prune on WNT signaling activation. Since Zhang and colleagues in the 2001 provided clear evidences that VEGF was strongly upregulated by the Wnt signaling pathway [Zhang, et al. 2001], here using the transgenic mouse pVegfr2-luc, in which the luciferase reporter is under control of murine Vegfr2 promoter, allowing to follow the WNT activation *in vivo* by enhancement of bioluminescence [Zhang, et al. 2004]. For this reason we created a double transgenic mouse model crossing our ML-h-Prune mouse (CD1) model with Vegfr2-Luc transgenic mouse (FVB/N) (fig 16 A). This line was backcrossed to FVB/N for 10 generations, generating albino ML-h-Prune/pVegfr2-luc transgenic animals (FVB/N). We validate h-Prune expression by both PCR (not shown) and WB within mouse epidermis.

In the epidermis of ML-h-Prune/Vegfr2-luc we found similar levels of expression of the human protein respect to the previous described ML-h-Prune transgenic mice (fig 16 B).



**Fig 16. ML-h-Prune/pVegfr2-luc double transgenic mice.** (A) Schematic representation of the cross pVegfr2-Luc and ML-h-Prune transgenic mice to obtain a double transgenic mice carrying both genes. (B) WB against Prune to validate the expression levels of the transgenic protein in the ML-h-Prune/pVegfr2-luc double transgenic mouse.

Moreover Western Blot analysis to better characterize the double transgenic mice ML-hPrune/pVegfr2-luc showed the same levels of ser9-p-GSK3 $\beta$ , total GSK3 $\beta$ , active  $\beta$ -catenin, total  $\beta$ -catenin and Cyclin D1 were detected in the ML-hPrune/pVegfr2-luc transgenic epidermis, as consequence of Wnt pathway activation (fig 17 A). On the other hand the protein amount of Notch pathways target gene shared the same trends in the epidermis of previous analyzed ML-h-Prune transgenic mice (fig 17 B). Finally since VEGFa is a downstream Wnt gene target, we expected an enhancement of bioluminescence *in vivo* in this ML-h-Prune/pVegfr2-luc. In figure 17 panel C we observed a substantial increase photons emission in ML-h-Prune/pVegfr2-luc (mice #6 - #10) with respect to the pVegfr2-luc (mice #1 - #5) transgenic animal. The difference of bioluminescence was higher between the 4<sup>th</sup> and 5<sup>th</sup> day of animal life, in according with the peak of h-Prune protein expression. After the 5<sup>th</sup> day of life, the differences in bioluminescence emission were gradually disappearing, according to what is reported in literature about the modified loricrin promoter ML [Waikel, et al. 1999]. Taken together, these results have revealed an important regulatory mechanism, exerted by h-Prune, within WNT pathway, inactivation of GSK3 $\beta$  proteins, that in turns could be exploited to an enhanced levels of Wnt pathway response.



**Fig 17. Characterization of the ML-h-Prune/pVegfr2-luc transgenic mice.** WB analysis for the Wnt pathway (A) and Notch pathway (B) related proteins showed the same protein levels in the epidermis of the previous ML-h-Prune single transgenic mice. (C) Photon emission in 5-days old ML-h-Prune/pVegfr2-luc respect to pVEGFR2-luc transgenic mice. The higher bioluminescence was a proof of the major activation of VEGFR2 promoter.

#### 4.9.H-Prune and human skin inflammatory diseases.

The results obtained above, showed that h-Prune is able to upregulate the Wnt canonical pathway. This leads to a decrease of Notch signaling activation revealed by decrease of its downstream Notch targets in the epidermis of ML-h-Prune transgenic mice. An increase in “Wnt/ $\beta$ -catenin” signaling is likely to be biologically significant, and has been associated with a wide number of skin malignancies including inflammation, cancer and psoriasis [Chan, et al. 1999, Moon, et al. 2004]. In particular in psoriatic skin was observed a strong nuclear localization of  $\beta$ -catenin in suprabasal layers [Hampton, et al. 2007] and also an increased expression of VEGF [Hardas, et al. 1996], both proteins related to the Wnt canonical pathways and were found upregulate in the ML-h-Prune transgenic mice. Moreover in the skin, Notch1 has a role in promoting keratinocyte differentiation and defects in the Notch pathway are thought to be involved in the pathogenesis of psoriasis. Notch signaling molecules are up-regulated in the basal and differentiating layers of

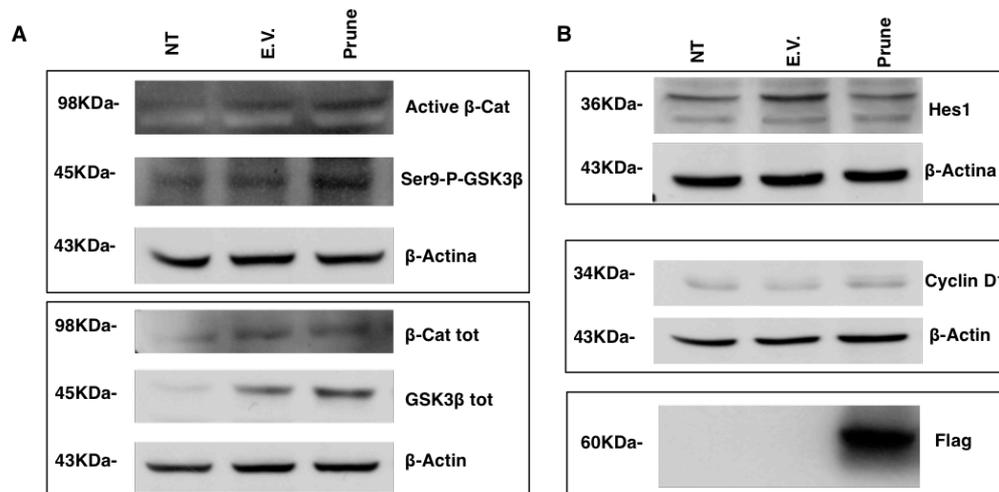
adult human epidermis, whereas they are under-expressed in disorganised proliferating epidermis [Rangarajan, et al. 2001, Thelu, et al. 2002].

Besides *H-PRUNE* is located in the 1q21.3 chromosomal region, 1Mb upstream the epidermal differentiation complex (EDC), a region which shows a remarkable density of genes that fulfil important functions in terminal differentiation of the human epidermis [Mischke, et al. 1996]. Linkage studies have shown in this region two susceptibility loci for skin inflammation disorders such as psoriasis [Capon, et al. 1999, Hardas, et al. 1996] and atopic dermatitis [Cookson, et al. 2001] for which no genes have been yet identified.

Taken together all these evidences, here we investigate the possible role of h-Prune may take part in human skin diseases like psoriasis which is known to occurs by a multi-genetic factors.

#### 4.10. In human keratinocytes h-Prune overexpression reproduces the Wnt signaling activation.

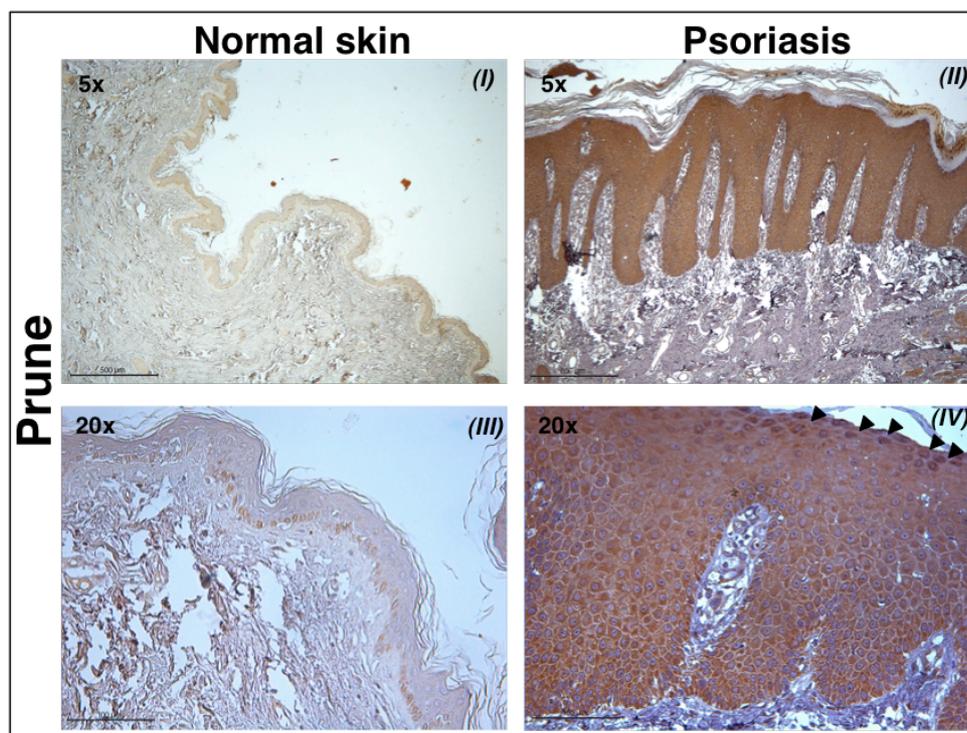
In order to verify whether h-Prune overexpression was able to reproduce the same effects observed in our mouse model, also in human context, we used a HACAT cell line as a model for in-vitro studies. H-Prune overexpression in HACAT keratinocyte cell line leads to an increase in the amount of the inactive ser9-P-GSK3 $\beta$  with the consequent increase of active  $\beta$ -catenin, while total GSK-3 $\beta$  protein quota was unvaried (fig 18 A), with a weak enhancement of downstream target cyclin D1 (fig 18 B) . This data confirm the Prune-mediated unbalance of Wnt pathway also in the human cell model.



**Fig 18. Altered Wnt and Notch downstream related protein upon H-Prune overexpression in HACAT cell line.** HACAT cells were transfected with pCS43x-Flag-h-Prune (Prune) and pCS43x-Flag-Empty Vector (E.V.). (A) In response to h-Prune overexpression high levels of active  $\beta$ -catenin, ser9-P-GSK3 $\beta$  and GSK3 $\beta$  quota were shown. No differences could be appreciated for total  $\beta$ -catenin. (B) A little decrease of Hes 1 protein occurred upon Prune overexpression, while no difference could be appreciated for Cyclin D1.

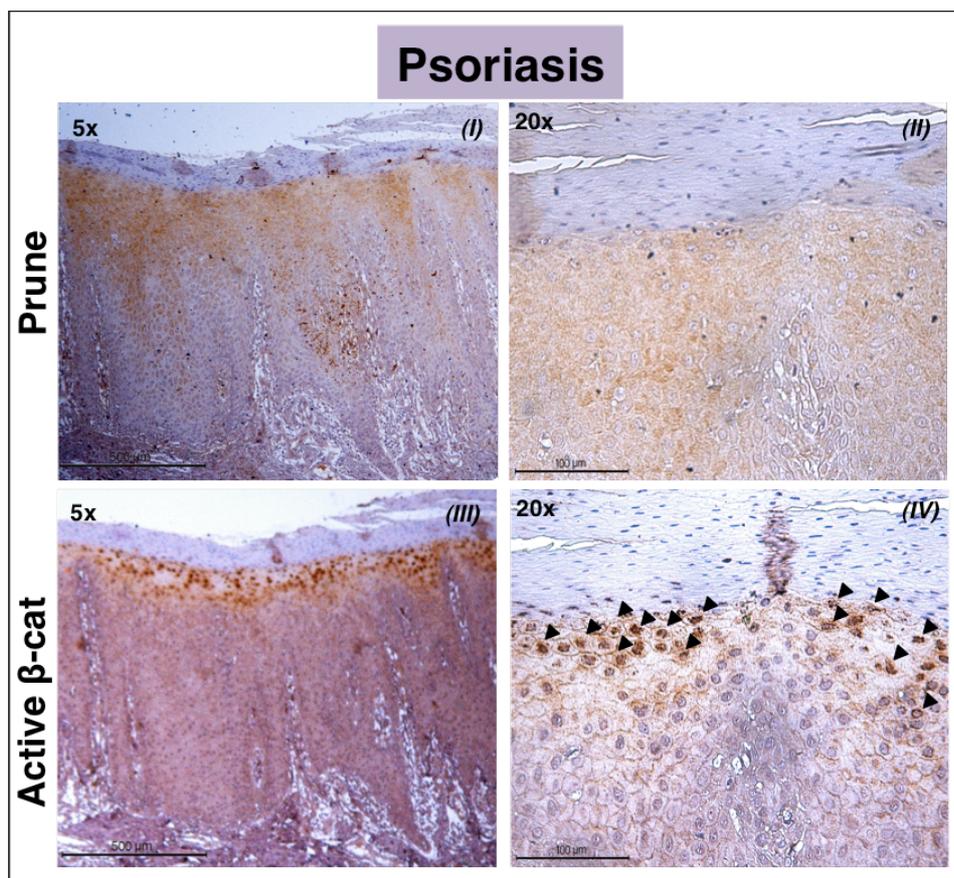
#### 4.11. Evidence of increased levels of h-Prune in human psoriatic samples and its correlation with $\beta$ -catenin nuclearization.

In order to verify the role of Prune in a context of human overgrowth skin disease, we decided to evaluate h-Prune protein levels by immunohistochemical analysis on a collection of human psoriatic samples and on an equal number of healthy skin samples, using an antibody against Prune (the same used for the mouse skin analysis). In all normal skin samples analyzed h-Prune showed a perinuclear and cytoplasmatic staining, and the signal is confined only in the basal layer of epidermis (fig 19 I and III). Interestingly almost all samples of psoriatic skin analyzed revealed an altered expression of h-Prune, showing a marked increase of immunoreactivity against the protein in all four layer of epidermis (fig 19 II and IV).



**Fig 19. Overexpression of h-Prune in human psoriatic samples.** Immunohistochemistry analysis of normal human skin and psoriasis sections with an antibody against Prune, showed that in normal skin samples h-Prune had a perinuclear and cytoplasmatic staining, and the signal was confined only in the basal layer of epidermis. h-Prune amount is strongly increased in psoriatic samples with a cytoplasmatic distribution. Nuclear staining for h-Prune was observed only in some cases and it was restricted to the more external layer (panel IV arrowheads).

The higher amount of Prune protein found in human psoriatic lesions compared to the normal samples provided a potential evidence for h-Prune involvement in this pathological disorder. Previous obtained data on our h-Prune mouse model showed that the high protein expression levels led to an increase in activation of Wnt pathway. In order to verify if the same event could occur in human psoriatic sample characterized by altered expression levels of Prune, we performed immunohistochemical analysis on serial slices of psoriatic skin samples with both an anti-Prune antibody and an antibody that specifically recognize the active  $\beta$ -catenin form. The figure 20 showed that high expression levels of h-Prune (fig 20 *I* and *II*), directly correlates with an increased of  $\beta$ -catenin nuclearization (fig 20 *III* and *IV*). A higher nuclear localization in the psoriatic lesion was already observed by Hampton et colleagues in 2007 [Hampton, et al. 2007]. Taken together our findings suggest a direct correlation between h-Prune overexpression and  $\beta$ -Catenin nuclearization in psoriatic samples, thus identifying h-Prune as a protein upregulated in psoriasis. At this time these evidences could be the base for future studies to identify a potential direct correlation between h-Prune and pathogenesis of psoriasis.



**Fig 20. H-Prune and active  $\beta$ -catenin staining in serial psoriatic sections.** Psoriatic skin sections screened for Prune and active  $\beta$ -catenin, showed a merge in the areas of expression of both proteins.  $\beta$ -catenin showed an higher nuclearization in the same areas of psoriatic epidermis that had higher expression levels of Prune. Arrowheads indicate the  $\beta$ -catenin positive nuclei.

#### 4.12. Discussion

H-Prune protein is known to date to stimulate cellular motility and metastasis process in a breast cancer model [D'Angelo, et al. 2004]. The role of h-Prune in the increased cell motility can be explained in part to h-Prune interaction with GSK-3 $\beta$  with which cooperatively works to regulate the disassembly of focal adhesions and promote cell migration [Kobayashi, et al. 2006].

In the present study we use a transgenic mouse model to investigate the role of h-Prune in the epidermis and its possible involvement in inflammatory/hyper-proliferative skin disorders highlighting the effects of h-Prune overexpression in unbalancing the Wnt and Notch signaling, two of the main pathways that regulate skin homeostasis [Artavanis-Tsakonas, et al. 1999, Gat, et al. 1998, Ito, et al. 2007, Lefort and Dotto 2004, Moriyama, et al. 2008, Zhang, et al. 2011].

We show Prune expression in both human and mouse skin to have a distinct pattern of expression in proliferating cells, thus suggesting its role in normal proliferation [Forus, et al. 2001]. High levels of h-Prune expression in the mice epidermis are correlated with a wrinkly skin with an epidermis moderately hyperplastic, with a thinner dermis and higher granular and spinous layer (fig 6 E). The failure of cell differentiation program is more evident in the upper layer of ML-h-Prune epidermis that shows an higher number of nucleated cells in this compartment respect to the WT mice which can be explained by the hyper-proliferation and delayed onset of differentiation in the suprabasal layers (fig 6 E, panel *III* and *IV*). Moreover in the ML-h-Prune transgenic mice skin can be noted disrupted epidermal microfibrils with less compact clusters of keratohyalin granule (fig 15 A) and filaggrin not located in the stratum corneum but rather spread in the thickness of suprabasal layers (fig 15 B).

All these epidermal alterations lead to defects in the skin barrier structure in the 6 days old transgenic animals (fig 7), that occurs two days after the maximum peak of expression of Loricrin promoter driven h-Prune transgene. This phenomenon has already been observed in other Loricrin-transgenic models highly expressed in the window of time between the 4<sup>th</sup> and 6<sup>th</sup> day of mice life [Waikel, et al. 1999].

Since GSK-3 $\beta$  is a h-Prune protein interactor, then the knowledge of GSK-3 $\beta$  as negative regulator of Wnt/ $\beta$ -catenin canonical signaling, we hypothesized at this time that the observed phenotypic alterations in the ML-Prune transgenic mice could be accounted to an inhibition of the negative regulation of GSK-3 $\beta$ , thus resulting on an enhancement of the downstream Wnt/ $\beta$ -catenin signaling. Wnt deregulation infact leads to an imbalance of proliferation and differentiation, often resulting in cancers [Reya, et al. 2001].  $\beta$ -catenin controls lineage selection of epidermal stem cells: high levels of  $\beta$ -catenin activation favours hair follicle differentiation, whereas inhibition of activation favours interfollicular epidermal differentiation [Fuchs, et al. 2004, Niemann and Watt 2002].

Hence we provide by WB and real time analysis the proof that h-Prune overexpression leads to Wnt pathway activation and inhibition of  $\beta$ -catenin degradation. This model should provide a basis for

future studies. Within the context in which  $\beta$ -catenin is activated, we observed an increased of basal cells proliferation in our transgenic mouse evidenced by an overexpression of keratin 14 (fig 6 F and H), which labels proliferative cells in the basal layer of the epidermis. Moreover we identify the h-Prune region able to turn on the Wnt pathway: C-Terminal portion (aminoacidic region 333-453), that is reported in literature to physically interact with GSK-3 $\beta$  [Kobayashi, et al. 2006], is able alone to upregulate the  $\beta$ -catenin-dependent transcriptional activity (TOP/FOP assay fig 9 B).

Interestingly, in the h-Prune transgenic skin we found elevated levels not only of inactive Ser-9-P-GSK3 $\beta$ , but also of GSK-3 $\beta$  protein quota. In literature is reported that when the Wnt pathway is active, Ser9-P-GSK3 $\beta$  is sequestered from the cytosol into multivesicular bodies, so that this enzyme becomes separated from its many cytosolic substrates, but in the same time is protected from protease degradation [Taelman, et al. 2010]. Preliminary data suggest us that Prune is able to increase the levels of Ser9-P-GSK3 $\beta$  that enter into multivesicular bodies to be protected from degradation. However further analysis are required to elucidate this issue. Our results are in keeping with these observations and provide the first direct evidence that h-Prune is essential for canonical Wnt signaling, but additional studies are required to clarify the role of this protein within this pathway. Among the target genes of the Wnt pathway that we found upregulated in the ML-h-Prune transgenic skin, there is the Notch ligand JAG1, which induces Notch1 signaling activation.

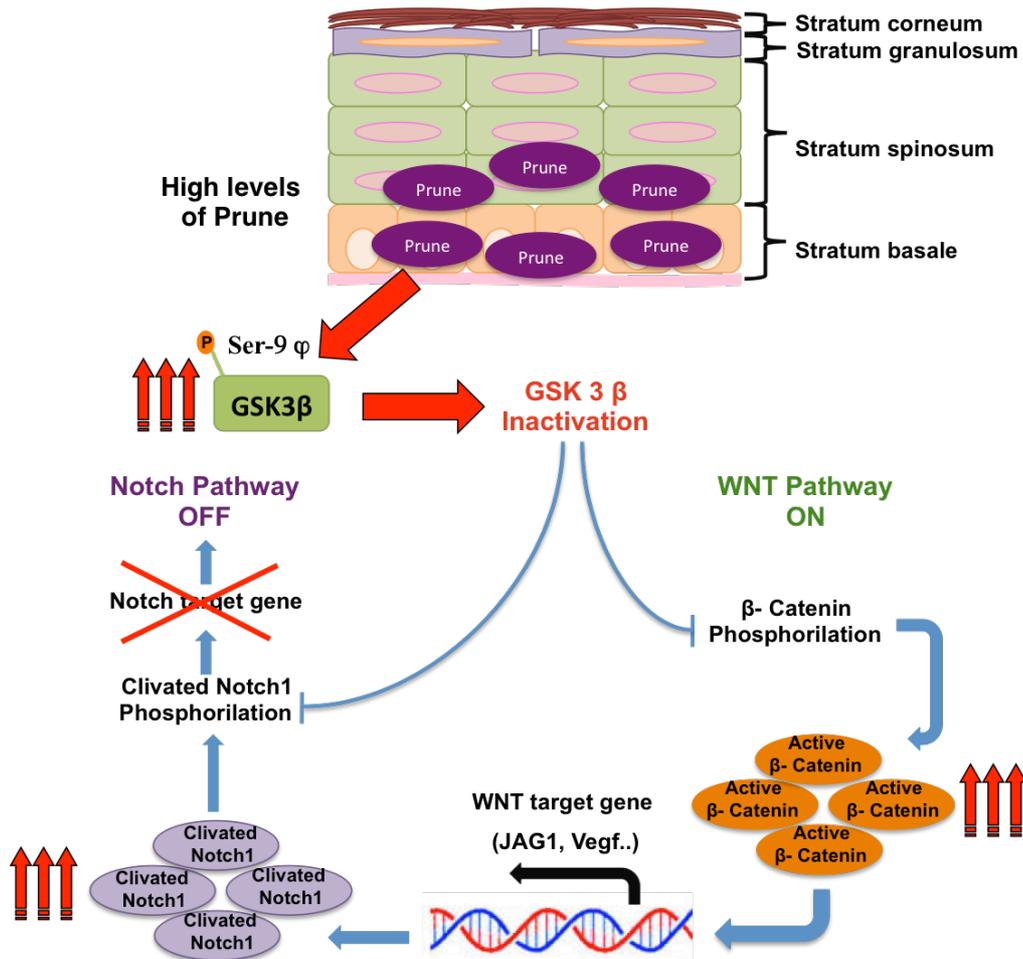
Notch1 is essential for coordination of keratinocyte growth and differentiation *in vivo* [Rangarajan, et al. 2001]. Notch signaling is required for commitment of basal keratinocytes to spinous cell differentiation at early stages of epidermal development [Blanpain, et al. 2006]. By contrast, postnatal ablation of Notch1 causes hyperproliferation of basal keratinocytes, suggesting that signaling from Notch1 is required for cell cycle withdrawal of the basal keratinocytes to promote terminal differentiation in the postnatal epidermis [Nicolas, et al. 2003, Rangarajan, et al. 2001].

Despite we found an high cleavage of Notch membrane receptor, the phenotype of the ML-h-Prune transgenic skin was similar to mouse model of Notch epidermal Knock Out present in literature showing an higher spinous and granular layers, high expression of cytokeratin 15 and disrupted kerathoyalin fibers [Moriyama, et al. 2008]. WB and Real time analyses revealed that the cleavage of Notch1 receptor in membrane and his internalization not carried to the pathway activation in transgenic epidermis (fig 12 and 13). We linked this observation with the increased GSK-3 $\beta$  inactivation found in the ML-h-Prune transgenic epidermis. GSK-3 $\beta$  is able to bind and phosphorylate NICD1 *in vitro* and attenuation of GSK-3 $\beta$  activity reduced the phosphorylation of NICD1 *in vivo* [Foltz and Nye 2001]. We verify this observation by a luciferase assay in HACAT cells: untreated cells transfected with h-Prune carried a reduction of the CBF mediated transcription that was comparable to the empty vector transfected cells treated with the potent GSK-3 $\beta$  inhibitor SB216763 (fig 14).

Moreover in this work for the first time we generate a useful tool to follow the Wnt pathway activation *in vivo*. Since VEGF is strongly upregulated by the Wnt signaling pathway [Zhang, et al. 2001] we

exploited this information to create the double transgenic mice pVegfr2-luc/ML-h-Prune that allow us to monitoring the downstream Wnt pathway effectors in vivo (fig 17 C).

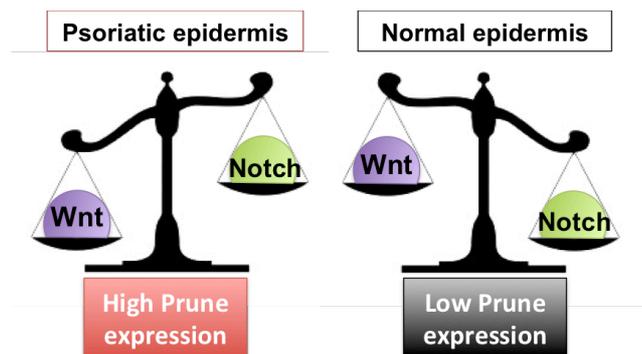
Hence taken together, these results have revealed a regulatory mechanism, exert by h-Prune, within WNT pathway, inactivation of GSK-3 $\beta$  proteins, that could be exploited in an enhancement of Wnt pathway and at same time in a downregulation of the Notch 1 pathway. The sum of both events led to a failure to basal keratinocytes differentiation program characterized by hyperproliferative and hyperplastic epidermis. Summarizing the results obtained so far, we hypothesized the following model to explain the action of Prune in the skin.



**Fig 21. Proposed model of prune overexpression into the skin.** Normally H-Prune is confined into basal layer of epidermis. As results of its overexpression occurs an increasing of GSK-3 $\beta$  phosphorylation rate which leads to inactivation of the latter. Phosphorylated GSK-3 $\beta$  is no longer able to phosphorylate  $\beta$ -catenin which escape from degradation, accumulates in the cells and enter into the nucleus to activate the Wnt target genes. Among the genes upregulated by Wnt pathway activation, there are also the Vegf and the Notch ligand Jagged1 (JAG1), that leads to an increasing in the amount of membrane Notch 1 clivation and internalization in the neighbour cells. At same time phosphorylated GSK-3 $\beta$  is no longer able to phosphorylates the intracellular clivated Notch1, then leading to the failure of downstream Notch signaling activation

These results lead us to investigate a possible involvement of h-Prune in a context of human overgrowth skin disease like Psoriasis.

The novelty of our work is the analysis of h-Prune protein levels in human psoriatic samples. For the first time we show elevated levels of Prune in this type of disease compared to the lower and specifically basal expression of the protein in the normal skin samples (fig 19). In such analysis we demonstrate furthermore a merge of h-Prune and nuclear  $\beta$ -catenin staining in serial psoriatic skin section (fig 20). We suppose that in the skin h-Prune works like a switch: in psoriatic samples high h-Prune levels correspond to increased Wnt signaling which led to the consequent Notch pathway inactivation. Conversely in the normal skin low levels of Prune do not turn on the Wnt pathway and the Notch downstream targets are normally active. Below we proposed a schematic representation of the balance between Wnt and Notch mediated by Prune in psoriatic skin. At this time these evidence will be the basis for additional studies in order to definitively infer a direct correlation of h-Prune into psoriatic disorders.



**Fig 22. Equilibrium between Wnt and Notch pathway mediated by Prune.** In the Psoriasis, the high expression of Prune protein leads to an increase in the Wnt pathway and consequent Notch pathway inactivation. Conversely in the normal skin low levels of Prune expression do not interfere in the normal differentiation program mediated by Notch.

## **5.CONCLUSIONS**

The purpose of our study was to understand the role of h-Prune in epidermal proliferation and keratinocyte-differentiation using a transgenic mouse model overexpressing the human protein into epidermis. We wanted first to characterize the phenotype of transgenic mice and to elucidate the molecular mechanism occurring in the skin of these animals. Moreover, we wanted to frame h-Prune function in the most frequent hyperproliferative disease of the skin, the psoriasis. In this regard we used the ML-h-Prune transgenic mice to conduct our studies.

In summary, our data demonstrate a functional role for h-Prune in regulating normal skin homeostasis, and implicate its altered expression in hyperproliferative skin diseases. Moreover we provide clear evidence of a regulatory mechanism in the balance of activation of the Wnt and Notch pathway mediated by Prune in the skin of transgenic animals. Moreover the double transgenic mouse pVegfr2-luc/ML-h-Prune that we have generated, was not only a useful tool to follow the trend of Wnt pathway *in vivo* but this animal should be a good candidate to test drugs able to impair the h-Prune influence on the Wnt pathway.

Finally, we assert an involvement of h-Prune in the pathogenesis of psoriasis, a disease for which a multigenes function is postulated. Future studies are required to clarify if Prune protein overexpression would be epistatic to the pathogenesis of psoriasis and/or if its levels are found increased following inflammation status of the damage tissue. If the first hypothesis occurs, h-Prune could represent a molecular target for therapy of hyperproliferative/inflammatory skin disorders including psoriasis.

## 6. Acknowledgment

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