

GENE EXPRESSION AND FUNCTIONAL STUDIES ON PSORIATIC EPIDERMIS

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"I know that I know nothing."

-Socrates

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numbers. Additionally, some unpublished data are presented.

- I. Katayama S, Skoog T, Jouhilahti EM, Siitonen HA, Nuutila K, **Tervaniemi MH**, Vuola J, Johnsson A, Lönnnerberg P, Linnarsson S, Elomaa O, Kankuri E, Kere J. 2015. Gene expression analysis of skin grafts and cultured keratinocytes using synthetic RNA normalization reveals insights into differentiation and growth control. *BMC Genomics*. Jun 25;16:476.
- II. **Tervaniemi MH**, Katayama S, Skoog T, Siitonen HA, Vuola J, Nuutila K, Sormunen R, Johnsson A, Linnarsson S, Suomela S, Kankuri E, Kere J, Elomaa O. 2016. NOD-Like Receptor Signaling and Inflammasome-Related Pathways Are Highlighted in Psoriatic Epidermis. *Scientific Reports*. Mar 15;6:22745.
- III. **Tervaniemi MH**, Söderhäll C, Siitonen A, Suomela S, Minhas G, Tiala I, Samuelsson L, Sormunen R, Saarialho-Kere U, Kere J, Elomaa O. 2012. Centrosomal localization of the psoriasis candidate gene product, CCHCR1, supports a role in cytoskeletal organization. *PLoS One*. 7(11):e49920.
- IV. **Tervaniemi MH**, Katayama S, Skoog T, Siitonen HA, Vuola J, Nuutila K, Tammimies K, Suomela S, Kankuri E, Kere J, Elomaa O. Psoriasis candidate gene product CCHCR1 affects several signaling pathways, supporting its function in centrosomes and P-bodies. *Submitted*.

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ABBREVIATIONS

AMP = antimicrobial peptide
BCC = basal cell carcinoma
CAMP = cathelicidin
CDS = cytosolic DNA sensor
CK = cytokeratin
DAMP = damage-associated molecular pattern
DAPI = 4',6-diamidino-2-phenylindole
DC = dendritic cell
DEG = differentially expressed gene
DET = differentially expressed transcript
DNA = deoxyribonucleic acid
EDC = epidermal differentiation complex
EGF = epidermal growth factor
EGFR = epidermal growth factor receptor
EKC = early passage keratinocyte
FACS = fluorescence-activated cell sorting
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GW = group-wise
GWAS = genome-wide association study
HaCaT = human immortalized keratinocyte
HEK293 = human embryonic kidney
IEM = immunoelectron microscopy
IF = immunofluorescence
IHC = immunohistochemistry
IFN = interferon
IL = interleukin
KC = keratinocyte
LD = linkage disequilibrium
LKC = late passage keratinocyte
MHC = major histocompatibility complex
NHEK = normal human epidermal keratinocyte
NLR = NOD-like receptor
PAMP = pathogen-associated molecular pattern
PC = principal component
PCA = principal component analysis

PCR = polymerase chain reaction
PBS = phosphate buffered saline
PRR = pattern recognition receptor
qPCR = quantitative PCR
RLR = RIG-I-like receptor
RNA = ribonucleic acid
RNA-seq = RNA sequencing
RT-PCR = reverse transcription PCR
SCC = squamous cell carcinoma
SG = skin graft
SNP = single nucleotide polymorphism
STAT3 = signal transducer and activator of transcription signal protein 3
STRT = single-cell tagged reverse transcription
TFE = transcript far 5'-end
Th = T helper
TLR = toll-like receptor
TNF = tumor necrosis factor
TSS = transcription start site
WB = western blotting

ABSTRACT

Psoriasis is a common skin disorder that is characterized by thickening of the most superficial layer of the skin, the epidermis, and accumulation of white blood cells, inflammation. The exact mechanism of how psoriasis develops is still unknown. Several gene expression studies have been conducted on psoriatic skin. Most of them, however, have focused on the expression in both the epidermis and dermis or were analyzed by microarrays. Here we used a novel approach to decipher the gene expression profile of the psoriatic skin, by utilizing a more specific and sensitive detection of transcripts by RNA sequencing (RNA-seq), implemented with an improved normalization method, and combined with samples that contain mainly the skin layer of interest: the epidermis. RNA-seq revealed more accurate expression profiles in different sample types that had varying amount of total mRNA per cell. Comparison with previous transcriptomics studies on psoriasis revealed that our approach provided more information about the transcriptional dysregulation in the epidermis. The expression profiling of epidermis highlighted the involvement of innate immunity and provided, for example, deeper understanding about the components of NOD-like receptor signaling pathway and inflammasome activation in keratinocytes. Some of the components have been associated with psoriasis in previous studies, yet the exact composition and activation mechanisms of inflammasomes have remained unclear. Our RNA-seq findings thus strengthen the role of keratinocytes as modulators of inflammation in the psoriatic lesions. The improved methods and focused analysis might help to pinpoint the most important pathways and functions, including broader knowledge in the involved components, in the psoriatic lesions. This, in turn, might improve the production of more specific treatments for psoriasis.

The psoriasis candidate gene *CCHCR1* is located in the major psoriasis predisposition locus *PSORS1*, contains a psoriasis-associated risk allele **WWCC*, and its gene product is expressed by the basal keratinocytes of the epidermis and has been shown to have an effect on cell proliferation and differentiation. The gene has two different transcription start sites and is able to encode for a peptide with a longer N-terminus from the transcript 1, which, however, depends upon a SNP that encodes for either tryptophan or stop codon, therefore either enabling or disabling the production of the longer protein. Here we presented association of the stop codon-encoding SNP (named as **Iso3*) with psoriasis in family trios.

We detected that CCHCR1 localizes at the centrosome and P-bodies, and reported isoform-specific effects on the localization of the P-bodies. Our experiments exhibited haplotype-specific effects of CCHCR1 also on cytoskeletal organization and cell proliferation; functions relevant to the pathogenesis of psoriasis. Furthermore, our results suggest that CCHCR1 might function in EGFR-STAT3 signaling and innate immunity, which strengthens the role of innate immunity in psoriasis even further. In addition, RNA-seq revealed isoform- and haplotype-specific effects on the expression profiles of different CCHCR1 cell lines. Interestingly, the most dramatic changes in gene expressions were observed in the isoform 3 -overexpressing cells but also the Non-risk and Risk haplotypes had antagonistic effects. The observation that CCHCR1 influences multiple cell signaling pathways may result from its possible role as a centrosomal P-body protein, which suggests a role in post-transcriptional regulation as well as a role in the regulation of cell cycle. Its exact function in these cellular compartments and effect in psoriatic lesions remains to be studied further.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease, characterized by red scaly plaques. The histological features include hyperproliferation and impaired differentiation of the keratinocytes, infiltration of inflammatory cells, and vascular changes. It has been considered as an autoimmune disease but recently it has been postulated whether it is in fact initiated by an abnormal response to pathogens in the skin, due to recent findings in genetics and transcriptomics, which support the importance of innate immunity. It is a multifactorial disease, including both genetic and environmental factors in the onset of the disease. The major susceptibility locus, *PSORS1*, includes the susceptibility genes *HLA-C*, with the risk allele **w6*, and *CCHCR1* that harbors the psoriasis-associated allele **WWCC*. This thesis addresses some of the questions regarding suitable methods of RNA sequencing on psoriatic samples, transcriptional profile of the psoriatic epidermis, and functional role of *CCHCR1* in psoriasis pathogenesis.

REVIEW OF THE LITERATURE

1. Skin

The skin covers the human body and is made up of multiple layers of tissue with ectodermal origin; it is our biggest organ. It is the primary interface between the body and the external environment and functions as a defensive barrier for the underlying muscles, bones, ligaments, and internal organs from physical and chemical trauma, pathogens, ultraviolet radiation, and prevents dehydration by regulating the transepidermal movement of water and electrolytes. Other functions include insulation, temperature regulation, sensation, and the production of vitamin D. It consists of three different layers (Figure 1): outermost layer is called the *epidermis*, underneath which locates the *dermis*, and the *subcutis* separates the skin from the inside of the body (Alberts et al., 2015). In this thesis, the function of the epidermis is on focus and we have used skin graft (SG) samples that contain less dermis than the usually used full-thickness biopsies (Figure 1).

1.1. Epidermis

Human epidermis (Figure 1) is a stratified squamous epithelium, consisting mainly (90-95%) of specialized epithelial cells: keratinocytes. It is separated from the dermis by the basement membrane, on top of which locates the basal layer of the epidermis (*stratum basale*). The lowermost part of the epidermis contains rete ridges, which are epidermal thickenings that extend downward towards the dermis. The epidermal stem cells and the keratinocytes that are able to proliferate are located at the basal layer and are connected to the basement membrane by hemidesmosomes. The keratinocytes leave the basal layer, at the same time withdrawing from the cell cycle, and differentiate during their journey through the epidermal layers towards the surface of the skin (Blanpain and Fuchs, 2006). Many factors affect the differentiation, one of the most important being the calcium gradient: the concentration of calcium is higher in the basal layer than in the following layer of the epidermis but again increases from there towards the surface (Leinonen et al., 2009). Melanocytes and Merkel cells are also located in this basal layer. The next layer is the spinous layer (*stratum spinosum*), in which the keratinocytes are connected to each other by desmosomes and under the microscope the cells appear spiky or “spinous”. Langerhans cells, among other immune cells, are located in this layer as well. On top of this layer the keratinocytes start producing lamellar bodies, which are secreted and form the

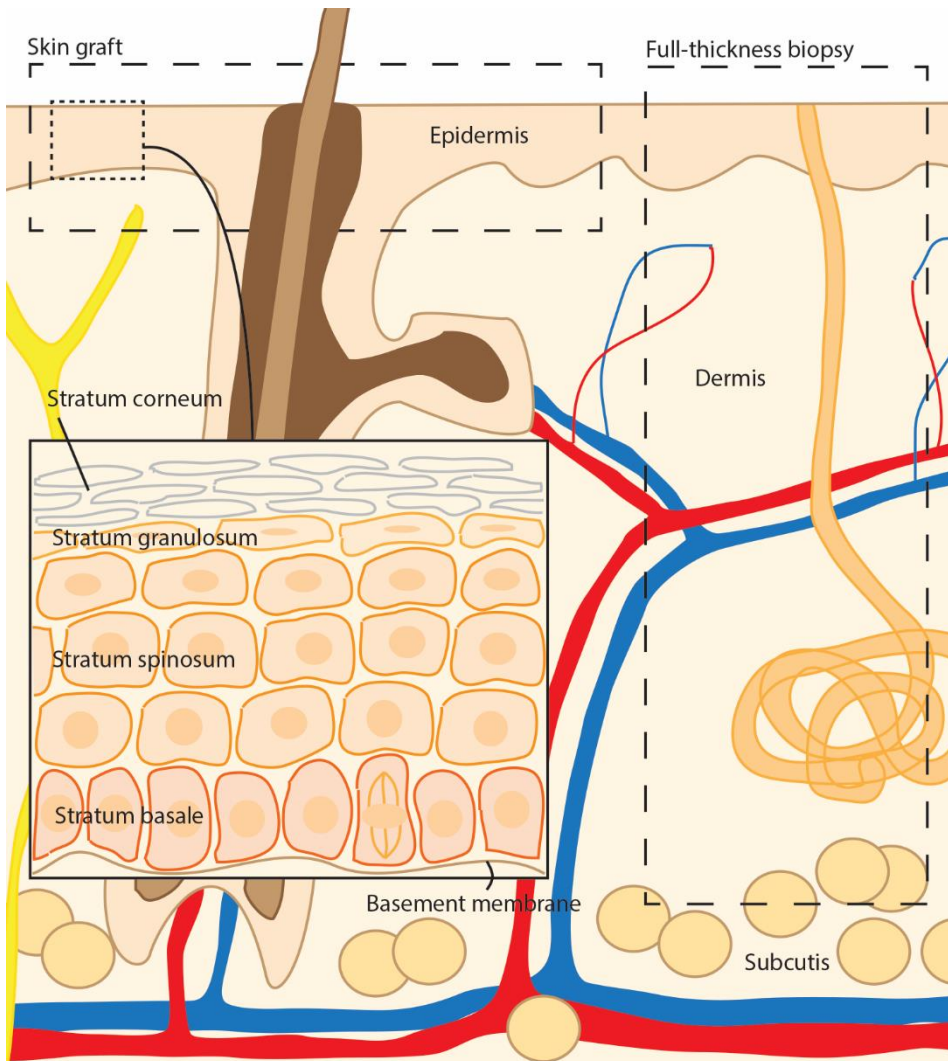


Figure 1 Structure of the skin. In the magnification illustrated the different layers of the epidermis with keratinocytes. In this thesis we focus on the epidermal gene expression, by using samples that are enriched by the epidermal skin (skin grafts). Most of the previous transcriptome studies on the skin have used full-thickness skin samples that contain both the epidermis and dermis.

lipid-rich extracellular barrier that protects from water loss from the inside and external factors from the outside. The next layer is the granular layer (*stratum granulosum*), where the keratinocytes continue producing the lamellar bodies, forming the barrier, and appear granular because their cytoplasm contains keratohyalin granules. The contents of the granules affect the differentiation, and

thus the keratinization of the cells. They also lose their nucleus and organelles and become non-viable corneocytes. Thick skin in palms and soles contains next the clear layer (*stratum lucidum*), which is absent elsewhere in the body. On the top of the skin locates the cornified layer (*stratum corneum*), in which the cells are connected to each other by corneodesmosomes and from which the flattened corneocytes desquamate, thus shed off, eventually. (Lowe et al., 2014; Nemes and Steinert, 1999; Rao et al., 1996)

Keratinocytes renew approximately within 6-10 weeks in the epidermis. Their differentiation is accompanied by large changes in gene expression, protein content, and cell morphology. Keratinocytes of the basal layer express large amounts of keratins 5 and 14 and are cylindrical in shape. During differentiation the cells start expressing keratins 1 and 10 and eventually become more flattened and tightly packed (Liu et al., 2007). Filaggrin, involucrin, and loricrin, among others, are markers of differentiated keratinocytes. The cornified envelope, a structure built of cross-linked proteins, lipids, and keratin bundles, starts to form beneath the cellular membrane and eventually replacing the cell membrane.

1.2. Dermis and subcutis

Dermis (Figure 1) is mainly composed of connective tissue, blood and lymphatic vessels, nerve endings, sweat and sebaceous glands, and hair follicles. It gives mechanical support for the skin and takes care of the needs of the epidermis. Connective tissue cells consist mainly of fibroblasts that secrete collagen and elastin fibers to their surroundings. In addition to fibroblasts, the dermis contains also white blood cells such as macrophages and lymphocytes. The uppermost (*stratum papillare*) layer of the dermis has fingerlike projections called papillae that extend toward the epidermis, intertwining with the rete ridges. The lower layer is called reticular dermis (*stratum reticulare*). Underneath the dermis is located the subcutis, which is mainly composed of loose connective tissue and fat cells, thus adipocytes. Its main function is to protect the body from hits and connect the skin to underlying tissues. (Breathnach, 1978)

2. Psoriasis

The word psoriasis comes from the Greek word psora “to itch”, which is quite an explanatory description of the disease. Psoriasis is a chronic inflammatory skin disease affecting 2–3% of people with European descent but occurring less in other populations such as in Asia and Africa (Roberson and Bowcock, 2010). The

histopathological changes in psoriatic skin include thickened spinous layer (*acanthosis*) with inward grown and elongated rete ridges (*epithelial buttressing*) and thickened cornified layer (*hyperkeratosis*). The keratinocytes within the psoriatic plaque proliferate excessively in the extended suprabasal layer (*hyperproliferation*), the granular layer is missing or abrupted (*hypogranulosis*), and the cells retain their nuclei in the cornified layer (*parakeratosis*). The eruption of the disease is associated with the infiltration of immune cells, such as neutrophilic leucocytes and macrophages, into the epidermis and dilation of papillary blood vessels in the dermis. The immune cells secrete proinflammatory cytokines, such as interleukins and chemokines, thus creating a “cytokine storm”. Moreover, psoriasis is characterized by increased turnover and altered terminal differentiation of the keratinocytes, including impaired degradation of desmosomes. The skin's barrier becomes dysfunctional and allows opportunities for external pathogens to enter the body (Mezentsev et al., 2014; Roberson and Bowcock, 2010).

Psoriasis is a complex multifactorial disease, with genetic factors affecting the susceptibility but also the environmental factors (such as smoking, psychophysical traumas, drugs, radiation, infection, obesity, and pregnancy) play a pivotal role in the onset of the disease (Elder et al., 2010). It is considered as a T-cell-mediated disease, addressing the activity of T-helper (Th)1, Th17, and Th22 cells and regulation by regulatory T cells (Treg) (Monteleone et al., 2011). It has been considered as an autoimmune disease but lately it has been postulated whether it is in fact initiated by an abnormal response to pathogens in the skin, due to genetic factors (Mattozzi et al., 2016; Mattozzi et al., 2012). Moreover, the recent findings in genetics and transcriptomics support the importance of innate immunity, which is known to be hyperactivated in psoriasis (Gudjonsson et al., 2009; Tsoi et al., 2012).

The patients also have an increased risk of comorbid diseases, such as psoriatic arthritis, metabolic syndrome (or components of it), cardiovascular disorders, anxiety and depression, non-alcoholic fatty liver disease, lymphoma, and Crohn's disease (Boehncke, 2015). It is also associated with psychological burden since the visibility of the disfigurements may impair the quality of life in the patients (Rapp et al., 1999).

2.1. Pathogenesis of psoriasis

Several functional characteristics of skin contribute to its effectiveness at maintaining homeostasis, despite the fact that it is regularly colonized by a variety of organisms. Antimicrobial peptides secreted by keratinocytes contribute to this maintenance by forming a shield against pathogens (Figure 2a). Several immune cell types are also resident in the skin. They maintain the steady-state immunity, and are ready to respond to a variety of stimuli. Balancing the defensive mechanisms is important for achieving homeostasis as disruption of any of these components contribute to the manifestation of dermatological diseases, such as psoriasis. (Stingl and Steiner, 1989)

2.1.1. Innate immunity

Innate immunity provides the first line of defence against infections. Most components of the innate immunity are present already before the onset of infection and are therefore not specific to a particular pathogen in the way that the adaptive immunity is. They include molecular components, such as antimicrobial peptides and the complement system, and phagocytic cells, such as macrophages and neutrophils that recognize classes of molecules characteristic to frequently encountered pathogens (Janeway, 2005). Skin and other epithelial surfaces provide anatomical and physiological barriers between the external environment and the inside of the body. Tight junctions, connecting the neighbouring cells, prevent easy entry by the potential pathogens. However, wounds in the skin create obvious routes for infection - psoriasis can be triggered by many factors, including injury and trauma (Köbner phenomenon), but also by infections and medications et cetera. Adaptive immune system plays an important role in the pathogenesis of psoriasis but the initial activation of the innate immune system is required at first. Moreover, recent studies also address the critical role of the innate immune system in psoriasis susceptibility but the medication strategies are currently aimed at the adaptive immune responses.

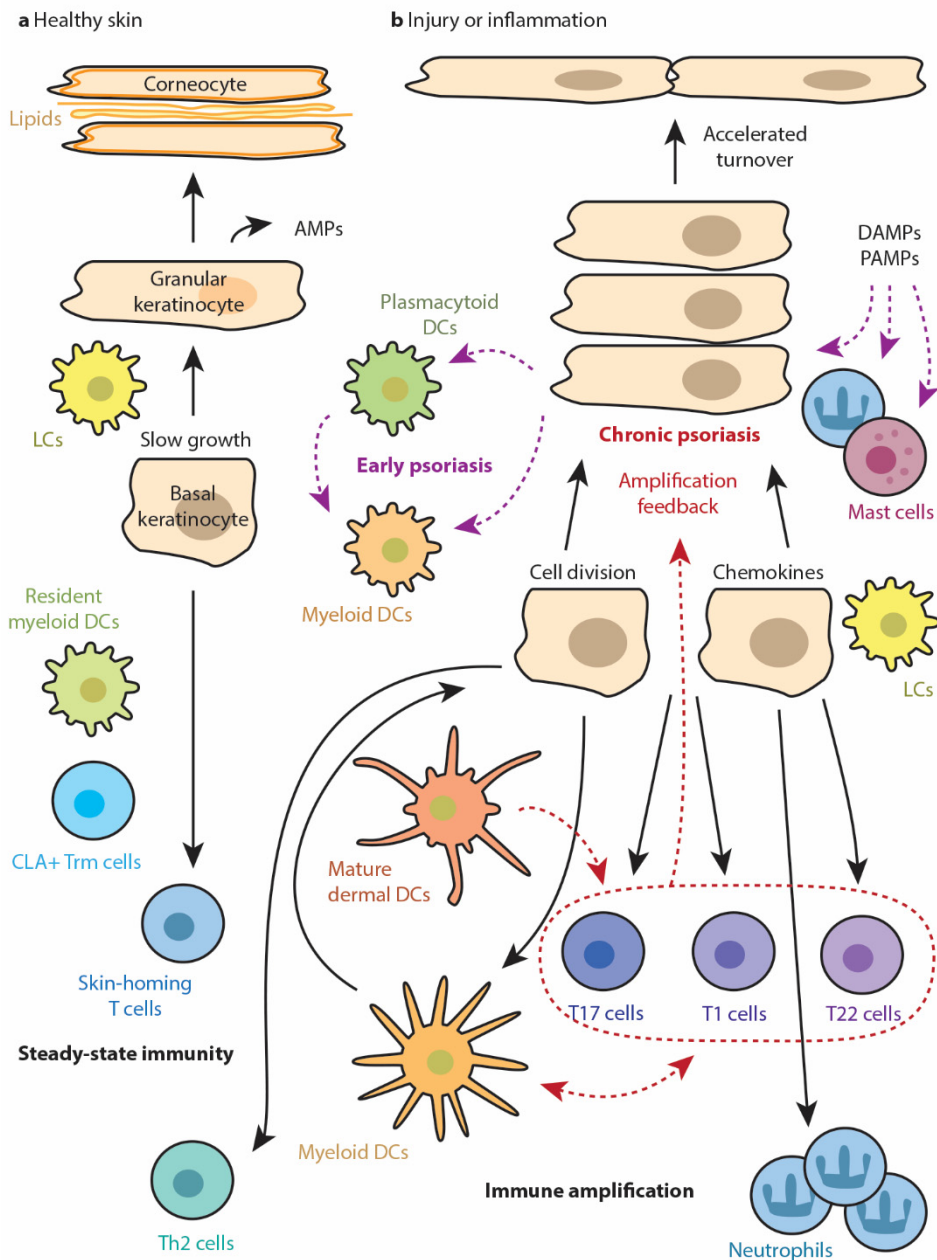


Figure 2 Inflammatory components of healthy and psoriatic skin. (a) Normal epidermis is formed by slowly differentiating keratinocytes. Antimicrobial peptides (AMPs) may be stored in the granular keratinocytes, including S100A family proteins, β -defensins, cathelicidin (CAMP), and lipocalin 2 (LCN2). The nuclei are lost as granular keratinocytes differentiate to corneocytes, and a cross-linked protein membrane structure termed the cornified envelope is formed, between which many layers of neutral lipids are deposited,

producing an effective water-impermeable barrier. Steady-state immunity is maintained by various immune cells: the epidermis contains Langerhans cells (LCs) and the dermis contains resident myeloid dendritic cells (DCs). There are also nonrecirculating cutaneous lymphocyte antigen (CLA)⁺ resident memory T cells (Trm cells) in the skin but keratinocytes constitutively synthesize CCL27 that attracts CCR10⁺ CLA⁺ skin-homing T cells into healthy skin for immune surveillance. **(b)** The epidermis participates also in innate or adaptive immune responses to triggers such as injury or infection. Keratinocytes proliferate in response to cytokines to accelerate loss of surface keratinocytes, eliminate pathogens, increase synthesis of innate effector molecules such as AMPs, and direct migration of new T cell subsets and other immune effector cells into the skin through production of chemokines. Pathways for initiation and maintenance of psoriasis are marked in purple and red. The keratinocytes within the psoriatic plaque proliferate excessively, the granular layer is abrupted, and the cells retain their nuclei in the cornified layer. Early disease (purple): CAMP released from keratinocytes (KCs) can bind to nucleic acids to activate plasmacytoid DCs to release IFN- α/β . CAMP/RNA complexes can also activate resident myeloid DCs to produce IL-12 and IL-23, key psoriatic cytokines. Extracellular DNA has been shown in the epidermis in association with neutrophil extracellular traps (NETs) and the role of mast cells has been implicated as well. Chronic psoriasis (red): The major pathogenic pathway in psoriasis occurs when mature dermal DCs and inflammatory myeloid DCs produce cytokines such as IL-23 and IL-12. These cytokines activate T helper and cytotoxic cells: T17, T1, and T22, to contribute to the cytokine milieu and further act on keratinocytes. Neutrophils are recruited to the epidermis and amplification loop retains chronic inflammation. Remade and modified from Lowes et al. 2014.

2.1.1.1. Barrier function

The stratum corneum maintains the uppermost epidermal barrier between the environment and the human body and is composed of terminally differentiated keratinocytes and extracellular lipids, such as ceramides (CER), cholesterol, and free fatty acids. The concentration and composition of ceramides is changed in psoriatic skin, which is suggested to affect the maintenance of skin barrier function and regulation of proliferation, differentiation, and apoptosis of keratinocytes (Borodzicz et al., 2016; Holleran et al., 2006). Corneodesmosomes also play a role in the pathogenesis of psoriasis; a reduced degradation of corneodesmosomal proteins (such as CDSN) in psoriatic lesions has been suggested, plausibly affecting the persistence of corneodesmosomes in the stratum corneum (Simon et al., 2008).

Tight junctions maintain the second physiological barrier in the stratum granulosum (Bazzoni and Dejana, 2002). Considering the impressively high amount of microbes constantly in contact with our skin, one of the functions of

the physical barriers is to segregate the immune receptors (and cells) from skin surface ligands to avoid activation of the receptors under non-pathologic conditions. Altered expression of several tight junction proteins have been observed in psoriasis lesional skin but their biological relevance in the pathogenesis of the disease is still unclear (Brandner et al., 2015).

2.1.1.2. Pattern recognition receptor families and inflammasomes

The activation of innate immunity relies on a limited set of pattern recognition receptors (PRRs) that recognize specific pathogen (PAMPs) or damage-associated molecular patterns (DAMPs) (Cao, 2016). The PAMPs are commonly present in microbes but not in mammals, whereas DAMPs are often cytosolic or nuclear molecules (DNA, RNA, S100 proteins etc.) that are released outside of the cell, e.g. upon exocytosis, injury, or necrosis. Activated PRRs trigger an inflammatory response leading to the efficient destruction of the invading pathogens, which include the secretion of cytokines, the induction of antimicrobial peptides (AMPs), pyroptotic cell death and the recruitment of phagocytic cells. The main PRR families are the Toll-Like receptors (TLRs), the NOD-Like receptors (NLRs), the RIG-I-Like receptors (RLRs), cytosolic DNA sensors (CDS), and the C-type lectin receptors (CLRs). They also play a central role in the activation of inflammasomes and autophagy (Schaefer, 2014). Inflammasomes are infection or stress-activated cytoplasmic protein complexes that consist of a NOD-like receptor (NLR) protein, caspase 1 (CASP1) (sometimes also CASP5), and the adaptor protein pyd and card domain-containing protein (PYCARD), which is the key component of the inflammasomes. The complex regulates the activity of CASP1, which is required for the processing and maturation of inflammatory cytokines; interleukins IL-1 and IL-18. The exact composition of the inflammasome depends on the activator that initiates its assembly (Latz et al., 2013). They have been studied extensively in immune cells, such as macrophages and langerhans cells, but many NLR inflammasome complexes are also expressed in human epithelial cells, such as keratinocytes.

The expression of several AMPs, whose expression can be triggered by the PRRs, is strongly increased in psoriatic plaques (Figure 2b) (Guttman-Yassky et al., 2011). The induction and early events of psoriasis have been suggested to begin with events involving the innate immunity (Figure 2b, purple). Dendritic cells (DCs) are professional antigen presenting cells that function as a link between the innate

and adaptive immune system, providing protection against commensals and invading pathogens. Keratinocytes, dermal fibroblasts, and immune cells secrete inflammatory cytokines under pathological skin conditions, which affects the activation and maturation of different DC populations, including Langerhans cells (LC) and dermal DC (Said 2015). Langerhans cells (LCs) are the only dendritic cells found in the epidermis during the steady-state and are considered as the first immunological barrier of the skin. Yet, their role in psoriasis has remained unclear. In psoriasis, injury or infection at the epidermis causes upregulated production of the AMP cathelicidin (CAMP) by keratinocytes (Lowes et al., 2014). CAMP can form aggregates with extracellular self-DNA that can activate TLR9 on the plasmacytoid DCs, thereby triggering interferon type I (IFN- α and - β) production (Lande et al., 2007; Nestle et al., 2005). CAMP/RNA complexes activate plasmacytoid DCs through TLR7, and myeloid DCs through TLR8, which are activated by type I IFNs as well. The myeloid DCs then activate T cells and thereby initiating the massive production of cytokines.

Transforming growth factor alpha (TGF- α) is induced in psoriasis and has been shown to affect the expression and function of TLR5 and TLR9 also in keratinocytes (de Koning et al., 2012b). Furthermore, the topical application of imiquimod, a ligand for TLR7 and TLR8, has been shown to induce psoriasis (Wu et al., 2004). TLR7 and TLR8 signaling leads to a type I IFN response and might affect the IL-23/IL-17 axis, which have been implicated in psoriasis as well (Ladoyanni and Nambi, 2005; van der Fits et al., 2009). In addition, CLR dectin-1 expression is increased in the epidermis of psoriatic lesions and its signaling stimulates immune cells to produce antifungal AMPs, which are highly expressed in psoriatic lesions (de Koning et al., 2010).

CAMP was also reported to function in keratinocytes, where it neutralizes cytosolic DNA (Dombrowski et al., 2011). Cytosolic DNA could induce IL-1 β secretion, thus triggering absent in melanoma 2 (AIM2) -dependent inflammasome activation, and is abundant in psoriatic lesions. AIM2 was also found upregulated in psoriatic lesions but CAMP could block the AIM2-dependent inflammasome activation. NLRP1-dependent inflammasome has been also recognized in psoriasis susceptibility (Ekman et al., 2014). Psoriatic keratinocytes have been shown to have increased sensitivity also to viral RNA intermediates, enhanced by IFN- α , by inducing expression of cytosolic innate RNA receptors, such as retinoic acid-induced gene-I (RIG-I) and MDA5 (Prens et

al., 2008). In addition, increased IL-18 expression has been reported by keratinocytes from psoriatic lesions (Rasmy et al., 2011). Neutrophils are crucial for clearing bacterial infections and extracellular DNA has been shown in the epidermis in association with neutrophil extracellular traps (NETs), which are networks of extracellular fibers that bind pathogens. The role of mast cells has been implicated as well (Kumar and Sharma, 2010), which further supports this role of innate immunity in the initiation of psoriasis.

2.1.2. Adaptive immunity

The adaptive immune system is composed of highly specialized cells and processes that eliminate or prevent pathogen growth, creating immunological memory. The major components of adaptive immunity are lymphocytes that produce antibodies, cytokines, and other molecules. It normally responds only to foreign antigens, thus being capable of self/nonself recognition. Adaptive immunity is not independent of innate immunity, which initially activates it and the two systems collaborate in eliminating the pathogen. Two major types of cells mediate the immune response: lymphocytes and antigen-presenting cells. Lymphocytes can be divided into two groups: B lymphocytes that express antibodies in their surfaces and T lymphocytes that express T cell receptors via which they recognize antigens that are bound to major histocompatibility complex (MHC) molecules. MHC I molecules can be presented basically by any nucleated cells (such as keratinocytes), whereas MHC II molecules are expressed by antigen-expressing cells (such as macrophages, B lymphocytes, and DCs) (Janeway, 2005).

T lymphocytes, especially T helper (Th) 17 and Th1 cells, are heavily present in psoriatic lesions (Lowe et al., 2007; Lowe et al., 2014). Moreover, tumor necrosis factor α (TNF- α) and inducible nitric oxide synthase (iNOS) -producing inflammatory DCs (TIP-DCs) and other inflammatory cells, such as macrophages, massively infiltrate to psoriatic skin (Harden et al., 2015a). Figure 2b illustrates (with red markings) a current pathogenic model of adaptive immunity in psoriatic lesions (Lowe et al., 2014). T cell priming is instructed by IL-12 and IL-23, which appear to be produced mainly from myeloid DC subsets in the skin. It was first revealed that IFN- γ -producing T cells, labelled as Th1 cells, are massively increased in psoriatic lesions (Lew et al., 2004). The T cells in psoriatic lesions also produce IL-17 (Th17) and IL-22 (Th22). There are also CD8+ T cell

populations in the lesions that make the same range of cytokines, which have been termed as Tc1, Tc17, and Tc22. In addition, $\gamma\delta$ T cells have been found to be IL-17-producing cells in psoriasis (Keijsers et al., 2014). It has also been suggested that mast cells and neutrophils, which are part of the innate immunity system, are the predominant cell types producing IL-17 in skin (Lin et al., 2011). Furthermore, skin is not merely a physical barrier but also a component of the lymphatic system, called as skin-associated lymphoid tissue (SALT). The chronic disease activity may be supported also by mature DCs (DC-LAMP+) that form cellular clusters with T cells in the dermis, a structure that can be considered as a form of induced SALT (iSALT) or tertiary lymphoid tissue (Egawa and Kabashima, 2011; Lowes et al., 2014).

Keratinocytes respond to the cytokines produced by each of these T cell subsets by upregulating the transcription of mRNAs encoding for numerous inflammatory products (Lowes et al., 2014; Nograles et al., 2008). Chronic T cell activation persists because the induced keratinocyte products have the ability to feedback on immune cells in the skin. The production of chemokines by keratinocytes is proposed to be important for the attraction of leukocyte subsets, such as neutrophils and myeloid DCs, which have relatively short life spans. Signal transducer and activator of transcription 3 (STAT3) is phosphorylated and activated in psoriatic lesions (Andres et al., 2013). It is important in the signaling pathways of several cytokines, such as IL-6, IL-10, IL-22, and IL-23 and STAT3 signaling can directly modulate epidermal hyperplasia (Tarutani et al., 2013). Moreover, it has been also reported in psoriatic lesions that IL-22 upregulates the expression of keratin 17 (KRT17), a hallmark and suggested autoantigen of psoriasis, in a STAT3-dependent manner (Zhang et al., 2012). However, as STAT3 is required for signaling through the IL-23R, it is essential for Th17 polarization (Harris et al., 2007) and it is possible that psoriasis-associated mutations in STAT3 (Tsoi et al., 2012) affect its effects on Th17 polarization. The main cell types involved in psoriasis and mentioned here above are collected in Table 1.

2.2. Genetics

Chromosomal regions harboring genetic association with psoriasis were initially entitled *PSORS* (psoriasis-susceptibility) loci. There are at least 15 different *PSORS* loci (Figure 3) that can be found from the Online Mendelian Inheritance in Man (OMIM), mainly identified through linkage analysis of multiply affected

psoriasis families and genome-wide association studies (GWAS) (Bowcock and Cookson, 2004; Ellinghaus et al., 2010; Harden et al., 2015b; Lowes et al., 2014; Marrakchi et al., 2011; Setta-Kaffetzi et al., 2014). The susceptibility gene or genes for most *PSORS* loci is still uncertain. The advances in methods and techniques in association studies, however, have enriched the understanding of the genetics of psoriasis.

Table 1 Cell types involved in psoriasis

Cell type	Location	Cytokines and AMPs
<i>Epidermal cells</i>		
Keratinocytes	Epidermis	IFN- γ , TNF- α , IL-1, IL-6, IL-8, IL-18, CAMP
<i>Stromal cells</i>		
Fibroblasts	Dermis	IL-1
<i>Dendritic cells</i>		
Myeloid DCs	Dermis, epidermis	IL-12, IL-23, TNF- α , IL-6
Mature DCs	Dermis	IL-23
Plasmacytoid DCs	Dermis	IFN- α and - β
TIP-DCs	Dermis	TNF- α , iNOS
Langerhans cells	Epidermis	
<i>Phagocytes and other related cells</i>		
Macrophages	Dermis	
Neutrophils	Dermis, epidermis	IL-17
Mast cells	Dermis, epidermis	IL-17
<i>Lymphocytes</i>		
Th17 cells	Dermis	IL-17A and F, IL-21, TNF
Th1 cells	Dermis	IFN- γ
Th22 cells	Dermis	IL-22
Cytotoxic T cells (Tc17, Tc1, Tc22)	Dermis	IL-17, IFN- γ , IL-22
$\gamma\delta$ T cells	Dermis, epidermis	IL-17

Located in the major histocompatibility complex (MHC) class I region (6p21.33), *PSORS1* is the most strongly associated locus for psoriasis predisposition (Trembath et al., 1997), especially for early onset psoriasis (Allen et al., 2005). The first gene identified in *PSORS1*, having significant association with psoriasis susceptibility, was *HLA-C* (Bowcock, 2005; Tiilikainen et al., 1980). Depending on the population being studied, the allele *HLA-Cw6* is found in about 4–16% of healthy controls and in 20%-50% of psoriasis cases, (Gourraud et al., 2014). *HLA-C* belongs to the MHC class I heavy chain receptors, which are present on almost all nucleated cells. They present intracellular peptides (both self and non-self peptides) to the immune system, therefore playing a key role in immune surveillance. MHCs are also critical for CD8+ T cell priming and subsequent cytolytic targeting of cells, which supports their important role in the pathogenesis of psoriasis (Harden et al., 2015b). The penetrance of the MHC-associated alleles is never 100% in psoriasis, even for monozygotic twins, which indicates the requirement of additional environmental or genetic modifiers for the development of specific T-cell receptor arrangements (Bowcock, 2005). However, the likelihood of *HLA-Cw6* driving the association of *PSORS1* has been controversial, plausibly due to the extensive linkage disequilibrium (LD) within the region. The region also includes the genes *CCHCR1* (alpha-helix coiled-coil rod homolog) and *CDSN* (corneodesmosin), both of which have been associated with psoriasis susceptibility as well (Asumalahti et al., 2000; Asumalahti et al., 2002; Tazi Ahnini et al., 1999).

Interestingly, *PSORS1* has been suggested to have an epistatic interaction with *PSORS4* locus on chromosome 1q21, which contains the epidermal differentiation complex (EDC) (Capon et al., 1999a; Capon et al., 1999b). The region harbors genes that are essential for the differentiation of keratinocytes and are divided into three families: cornified envelope precursor proteins (loricrin (LOR), involucrin (IVL), small proline-rich proteins (SPRPs), and late cornified envelope proteins (LCE)), keratin filament-binding proteins (filaggrins (FLs), trichohyalin (TCHH), repetin (RPTN), hornerin (HRNR), and cornulin (CRNN)), and S100 calcium-binding proteins (some of which also act as chemokines and are upregulated during skin inflammation) (Chen et al., 2009; Zhao and Elder, 1997).

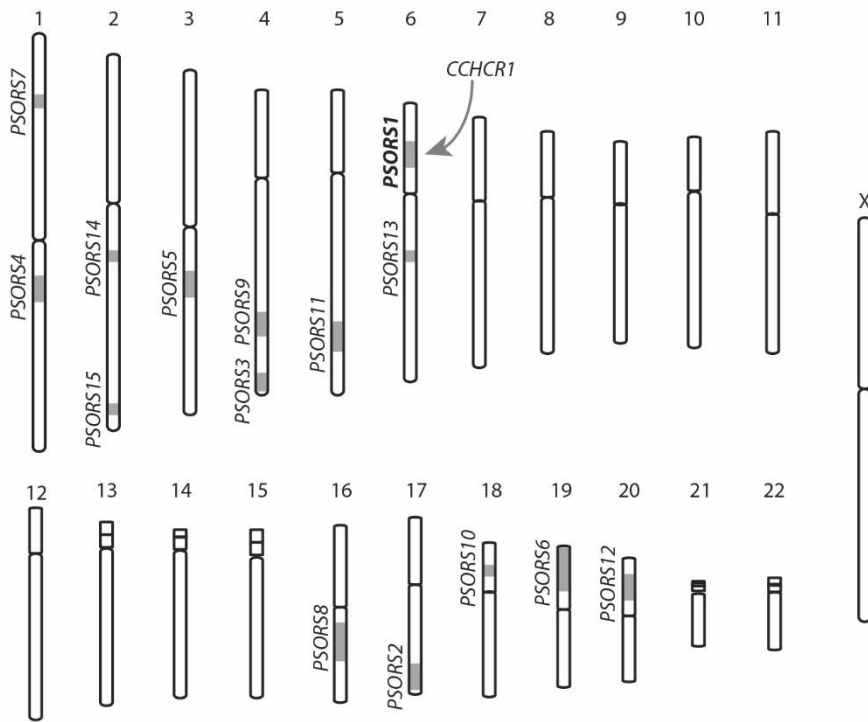


Figure 3 Chromosomal locations of the PSORS loci. The numbers represent the chromosomes. The identified loci are illustrated in grey. Information of the loci are collected from Bowcock and Cookson, 2004 and Online Mendelian Inheritance in Man (OMIM).

2.2.1. Psoriasis candidate gene *CCHCR1*

The gene *CCHCR1* (Coiled-Coil α -Helical Rod protein 1) is located in *PSORS1* (Asumalahti et al., 2000; Asumalahti et al., 2002). The gene has at least two transcription start sites (TSSs) (Figure 4) and encodes for several transcripts and at least 3 different protein isoforms. The transcripts for the isoforms 1 and 2 are transcribed from the second TSS (exon 1b), whereas the transcript starting from the first TSS can encode only for the isoform 3, which has therefore a shorter N-terminus. The protein encoded by the gene, *CCHCR1*, is predicted to have α -helical coiled-coil rod domains and possibly a leucine zipper motif, but it exhibits little homology with other known proteins; the strongest homologies are with various myosins (Asumalahti et al., 2000). Noteworthily, it was originally identified that the *CCHCR1* gene shares also some structural homology to

trichohyalin (TCHH); a gene located at the PSORS4 locus and encoding for an intermediate filament-associated protein that is mainly expressed in the granular layer of the epidermis (Guillaudeux et al., 1998).

2.2.1.1. Genetic associations with psoriasis and other diseases

The location at the chromosomal region showing the strongest associations in genome-wide association studies (Zhang et al., 2009), suggests *CCHCR1* as a plausible psoriasis susceptibility gene in addition to *HLA-C*. Its role and function

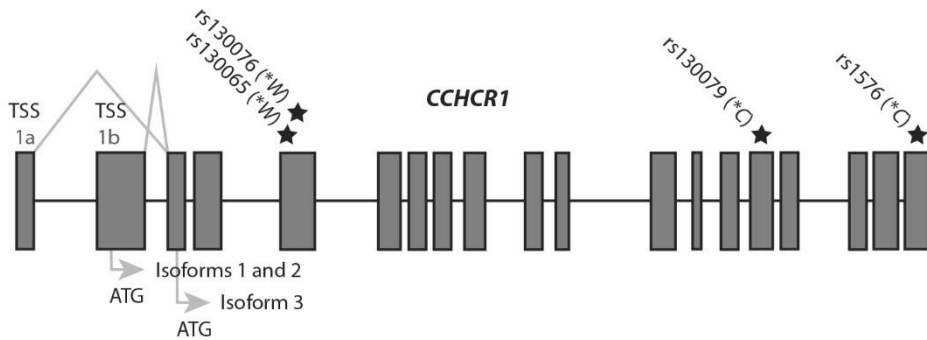


Figure 4 Structure of the *CCHCR1* gene. The gene contains 18 exons (not in scale), with alternating first exon and transcription start sites (TSS, exons 1a and 1b). The exon 1b contains a codon ATG and is able to encode for *CCHCR1* isoforms 1 and 2 that are longer by their N-terminus than isoform 3, which is translated from ATG in exon 2. The stars show the positions of the **WWCC* risk haplotype SNPs in exons 4, 14, and 18.

in the pathogenesis of psoriasis, however, is still unclear. *CCHCR1* is highly polymorphic and previous studies have identified an allele, *CCHCR1***WWCC*, which is associated with psoriasis susceptibility in several populations (Asumalahti et al., 2000; Asumalahti et al., 2002; Chang et al., 2006). The allele **WWCC* is composed of four nonsynonymous SNPs along the coding region of *CCHCR1* (Figure 4). In the non-risk haplotype these SNPs encode for amino acids: R (rs130065), R (rs130076), G (rs130079), and S (rs1576), whereas in the risk haplotype they are W, W, C, and C. The SNPs rs130065, rs130076, and rs1576 have been associated primarily with early onset psoriasis (type I) (Allen et al., 2005; Chang et al., 2005; Chang et al., 2004; Prieto-Perez et al., 2015). SNPs within *CCHCR1* have been associated also with nevirapine-induced rash in HIV-infected patients, multiple sclerosis, and type 1 diabetes susceptibility (Chantarangsu et al., 2011; Cheung et al., 2011; Lin et al., 2015).

2.2.1.2. *Function*

The function of *CCHCR1* has not been extensively studied but the protein has been suggested to localize in the cytoplasm, nucleus, and mitochondria and to regulate various cellular functions, including steroidogenesis, proliferation, and differentiation (Corbi et al., 2005; Sugawara et al., 2003; Suomela et al., 2003; Tiala et al., 2007). In addition, a recent study localized the protein *CCHCR1* to the P-bodies, which are sites for mRNA metabolism (Ling et al., 2014). The predicted structure of the *CCHCR1* protein with risk allele **WWCC* differs from the wild-type by a shorter first alpha-helical domain, which possibly affects the properties of the protein.

CCHCR1 interacts with steroidogenic activator protein StAR, via which it regulates the synthesis of steroids from cholesterol in mitochondria (Sugawara et al., 2003; Tiala et al., 2007). Moreover, the gene expression for lipid biosynthesis has been shown to be decreased, already in the non-lesional psoriatic skin, which supports the role of altered lipid metabolism in the pathogenesis of psoriasis (Gudjonsson et al., 2009). *CCHCR1* has been shown to interact also with RNA polymerase II subunit 3 (RPB3), for which it functions as a cytoplasmic docking site, thereby controlling myogenic differentiation. (Corbi et al., 2005). In addition, it has been shown to interact with enhancer of mRNA-decapping protein 4 (EDC4), in the P-bodies (Ling et al., 2014). Several other possible interacting partners for *CCHCR1* have been identified by yeast two-hybrid experiments, including: protein kinase C gamma (PRKCG), TNF receptor-associated factor 4 (TRAF4), DNA polymerase delta subunit 2 (POLD2), inhibitor of nuclear factor kappa-B kinase subunit gamma (IKBKG), transforming acidic coiled-coil-containing protein 3 (TACC3), and with proteins from several viruses and bacteria, such as Epstein-Barr virus (EBV), human papillomavirus (HPV), and hepatitis C virus (HCV) (Gomez-Baldo et al., 2010; Lim et al., 2006; Ling et al., 2014; Wang et al., 2011; Xu et al., 2002).

The expression of *CCHCR1* in psoriatic lesions differs from healthy skin or other hyperproliferative skin disorders (Suomela et al., 2003; Tiala et al., 2007). *CCHCR1* is expressed in the keratinocytes at the basal layer of the epidermis, in healthy skin. In psoriatic lesions, however, *CCHCR1* is expressed also in the suprabasal keratinocytes above the tip of the dermal papillae, while the hyperproliferation marker Ki67 is expressed in the rete ridges, where the expression of *CCHCR1* is

less prominent (Asumalahti et al., 2002; Suomela et al., 2003). In other hyperproliferative inflammatory skin disorders, such as chronic eczema, chronic skin ulcers, and lichenoid chronic dermatitis, the expression pattern is absent and the expression resembles normal skin. The overexpression of CCHCR1 has been shown to affect keratinocyte proliferation in transgenic mice, in which the most evident effect occurred after wounding or treatment with 12-O-tetradecanoyl-13-acetate (TPA). The wound healing was delayed and TPA-induced epidermal hyperproliferation was less pronounced in mice with the overexpression of the *CCHCR1*WWCC* risk allele (Tiala et al., 2008). Moreover, the overexpression of CCHCR1 had an effect on the expression of several genes relevant in psoriasis pathogenesis, these including keratins 6, 16, and, 17 (*Krt6/16/17*), and genes of the epidermal differentiation complex region on the *PSORS4* locus (1q21), such as S100 calcium binding protein A1 (*S100A*) and small proline-rich protein (*Sprp*) (Elomaa et al., 2004).

CCHCR1 is expressed also in different cancers of epidermal origin, such as adenocarcinoma of the lung and breast (Suomela et al., 2003). The hyperproliferation marker Ki67 was not expressed by the same cells, as in the case of psoriasis. The expression of CCHCR1 has been studied also in non-melanoma skin cancers: squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) (Suomela et al., 2009). It was expressed especially in Ki67 positive proliferating cells of the tumors. Furthermore, when compared with normal cultured keratinocytes the mRNA expression was upregulated in SCC cultures. Similar increase in *CCHCR1* expression has been detected in neoplastic cervical high-grade squamous intraepithelial lesions (Pacholska-Bogalska et al., 2012). Moreover, the strongest CCHCR1 expression in SCCs and BCCs was detected in areas positive for epidermal growth factor receptor (EGFR). EGFR and related receptors are well known markers in several solid tumors and their expression and signaling are implicated in pathogenesis of psoriasis as well; especially, many of the EGFR ligands are overexpressed in psoriatic epidermis (Schneider et al., 2008; Yoshida et al., 2008a). The constant stimulation of EGFR is suggested to constitutively activate the signal transducer and activator of transcription signal protein 3 (STAT3), resulting in effects on skin via alteration of biological processes in keratinocytes, such as proliferation, differentiation, and apoptosis (Chan et al., 2008; David et al., 1996; Sano et al., 2008). In addition, epidermal growth factor (EGF) induces CCHCR1 expression in keratinocytes (Tiala et al., 2007), whereas interferon- γ (IFN- γ) does the opposite (Suomela et al., 2003).

2.3. Transcriptomics

Several gene expression studies have been conducted on psoriatic skin, providing evidence on the transcriptional changes in the lesions but also already in the non-lesional skin of a patient with psoriasis. Gene expression studies that have compared the expression in psoriatic lesional skin with control and/or non-lesional skin by microarrays have revealed numerous differentially expressed genes (DEGs) (Bowcock et al., 2001; Gudjonsson et al., 2010a; Gudjonsson et al., 2009; Mitsui et al., 2012; Reischl et al., 2007; Suarez-Farinas et al., 2010; Tian et al., 2012; Yao et al., 2008; Zaba et al., 2009; Zhou et al., 2003). RNA sequencing (RNA-seq) has provided a new and more specific alternative to microarrays. However, only a few RNA-seq studies on gene expression in psoriatic skin have been published thus far (Jabbari et al., 2012; Li et al., 2014) and a couple that are limited to the microRNA and long non-coding RNA transcriptomes (Gupta et al., 2016; Joyce et al., 2011). Most of the previous psoriasis transcriptome analyses have used full-thickness skin samples but one study focused on microdissected samples that separated the epidermis and dermis (Mitsui et al., 2012).

Keratinocytes change their gene expression profiles during the development of a lesion, due to the stimuli by inflammatory factors. Also the invading inflammatory cells affect the gene expression profile of the psoriatic epidermis. Most of the inflammatory cells, however, reside in the dermis. Meta-analysis derived (MAD) analysis (Tian et al., 2012) that combined 5 different microarray data sets of full-thickness samples, comparing lesional skin expression with non-lesional skin, highlighted atherosclerosis signaling, lipid metabolism, and cardiovascular development, strengthening the similarity of the disease with other comorbid diseases, such as metabolic syndrome and cardiovascular disease. The separate microarray analysis of epidermal and dermal sections of psoriatic skin, in comparison with non-lesional skin samples, revealed local production of C-C motif chemokine ligand 19 (*CCL19*) and its receptor *CCR7* in psoriatic dermal lymphoid aggregates (Mitsui et al., 2012). The study also illustrated the presence of mature DC markers LAMP3/DC-LAMP and CD83 in the aggregates, proposing lymphoid organization via *CCL19/CCR7* in lesional psoriatic dermis.

The RNA-seq analysis conducted with full-thickness samples of psoriatic lesions and healthy controls revealed marked differences in sensitivity, when compared with analysis by microarray. Transcripts identified only by RNA-seq had much

lower expression than those also identified by microarray (Li et al., 2014). It is commonly observed that the DEGs differ greatly across experiments, partially due to variations introduced in the microarray or RNA-seq experimental pipelines. RNA-seq increased the detection of differentially expressed transcripts (DETs) enriched in immune system processes. Weighted gene co-expression network analysis with the shared genes from microarray and RNA-seq highlighted epidermal differentiation genes, lymphoid and/or myeloid signature transcripts, and genes induced by IL-17 in keratinocytes in this analysis. The analysis also emphasized the significant down-regulation of dermally expressed genes in psoriatic biopsies, which was suggested to result from technical artefact caused by the expansion of the epidermal compartment, emphasizing the influence of tissue architecture in expression analyses.

3. RNA sequencing

RNA-seq has become a widely used method for transcriptome studies. It uses deep-sequencing technologies that provide a far more precise measurement of the levels of transcripts and their isoforms than other methods (Wang et al., 2009). When compared with microarrays, the main advantages of RNA-seq are: sensitivity, ability to detect splice variants, transcription start sites (TSS), and intergenic transcripts.

The transcriptome is the whole pool of transcripts in a cell or a tissue and it varies between different tissues or cell types and specific developmental stages or physiological conditions. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents and pathways of cells and tissues. It is also important for understanding the pathogenesis of a disease. The key aims of transcriptomic studies are: to decipher the expression profile of all species of transcripts, including mRNAs, non-coding RNAs, and small RNAs; to determine the transcriptional structure of genes and the genome, in terms of their start sites, 5' and 3' ends, splicing patterns, and other post-transcriptional modifications; and to quantify the changes in expression levels of each transcript during development and under different conditions.

Different RNA-seq methods have different advantages (Hrdlickova et al., 2016). In this thesis we have used a highly multiplexed and strand-specific method that was originally designed for single-cell RNA 5' end sequencing (single-cell tagged

reverse transcription, STRT; Figure 5) (Islam et al., 2012). Since the epidermis of the skin is very thin, the amount of RNA extracted is also very modest. Therefore, this method, which is designed for minute amounts of RNA, is very suitable for our samples. In addition, the early bar-coding strategy reduces costs and time. Compared with previous methods, this one is unsuitable for the detection of alternatively spliced transcripts but is more suitable for large-scale quantitative analysis, as well as for the characterization of transcription start sites, yielding clues for gene regulation.

3.1. Normalization

Normalization can be described as the removal of systematic experimental bias and technical variation to improve the identification of changes in the transcript expressions, across different conditions (Meyer et al., 2010). There are several normalization methods published, such as median and quantile normalization methods and probably the most well-known is the reads per kilobase of transcripts per million mapped reads (RPKM) normalization (Mortazavi et al., 2008). Another strategy aims to represent the “global fold-change” by introducing a scaling factor called trimmed mean of M-values (TMM) (Robinson and Oshlack, 2010), resulting in samples of similar total expression, which may not be biologically correct. All of the methods mentioned above, depend on the global gene expression. The method used in the RNA sequencing performed in this thesis, applies normalization by RNA spike-in (Katayama et al. 2012, Islam et al., 2011, Islam et al. 2012).

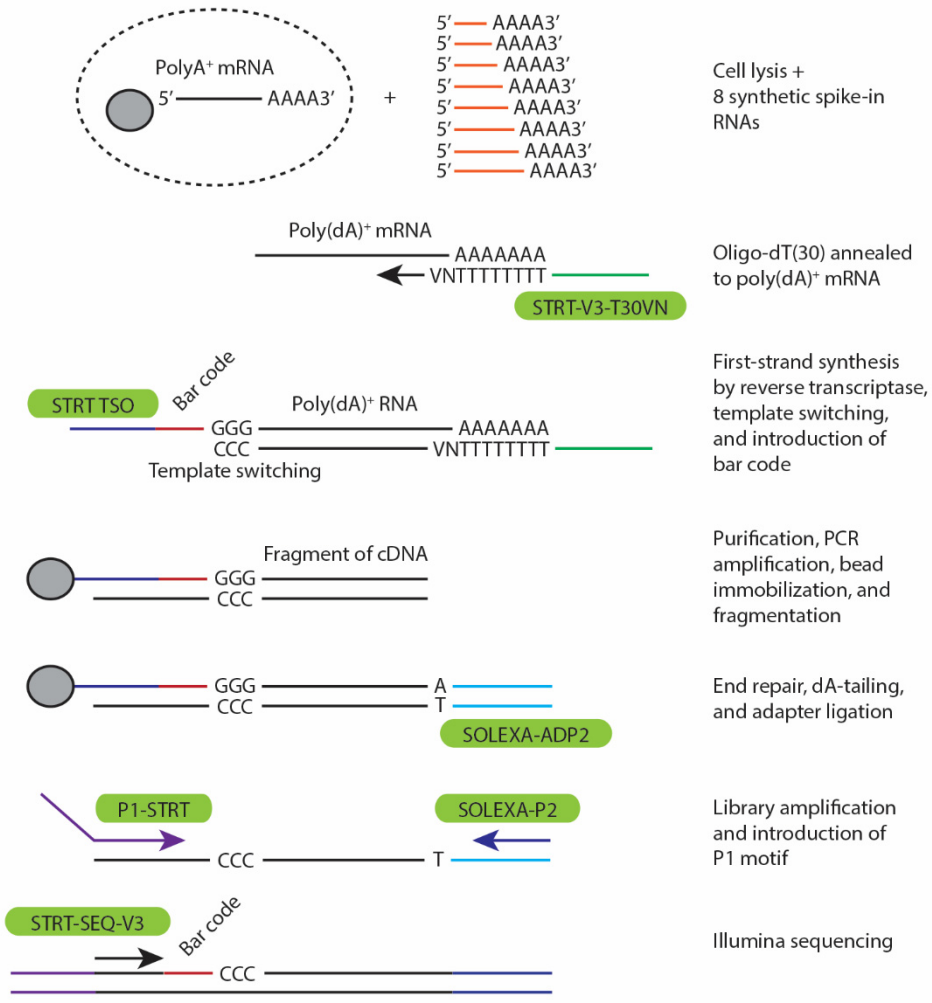


Figure 5 Schematic overview of the STRT RNA sequencing method with RNA spike-in normalization. The tissues/cells are lysed, RNA spike-in molecules added, and mRNAs converted to cDNA. By using a template-switching mechanism; a bar code and an upstream primer-binding sequence are introduced simultaneously with reverse transcription. All the cDNAs are pooled and prepared for sequencing - preparation including: fragmentation, adapter ligation, and PCR amplification. SOLEXA refers to the sequencing instrument used originally; presently, the most commonly used platform is Illumina. Remade and modified from Islam et al., 2012.

AIMS OF THE STUDY

The main aim of this study was to identify the causative elements behind psoriasis. Thus, the thesis is focused on identification of aberrant signaling pathways in psoriatic epidermis and studies with psoriasis candidate gene *CCHCR1*.

The aim of the first RNA-seq study was to improve RNA-seq methods with which to investigate samples with varying amounts of poly A+ RNA and to identify transcriptional differences between different keratinocyte sample types: tissue samples, cultured keratinocytes, and keratinocyte cell line. The improved method was applied to the psoriasis study, where the aim was to focus on differences in transcriptome profiles of healthy control, non-lesional psoriatic epidermis, and lesional psoriatic epidermis. A database survey (NCBI's GenBank) suggested that *CCHCR1* has alternative transcripts 1, 2, and 3, at least, of which 1 is the longest and 3 the shortest. We were interested in the effects of *CCHCR1* on transcriptional regulation, as many of its already known functions implicate a role in transcriptional regulation. Here we focused on the effects of the *CCHCR1* protein isoforms encoded by the longest and shortest transcripts.

The specific aims of this thesis were to:

1. Identify transcriptome and gene expression profiles of psoriatic healthy/lesional vs control skin (I, II, Figure 6).
2. Investigate the isoform/haplotype specific function of *CCHCR1* (III), its effects on transcription and signaling pathways, and relevance in psoriasis (IV) (Figure 7)

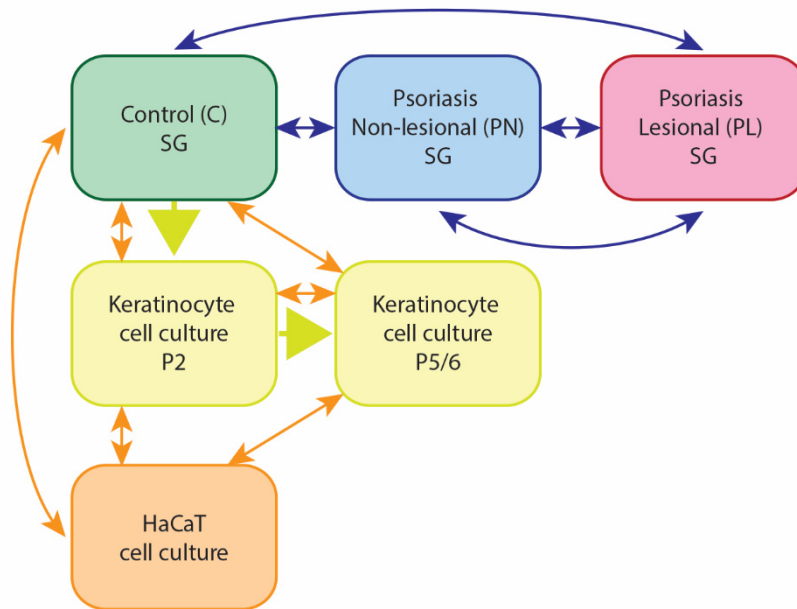


Figure 6 RNA-seq: keratinocyte and psoriasis study samples. The top row represents the psoriasis samples and the blue arrows indicate which samples were compared together. The orange arrows indicate the comparisons in the keratinocyte study. The control samples were used in both of the studies. SG = skin graft.

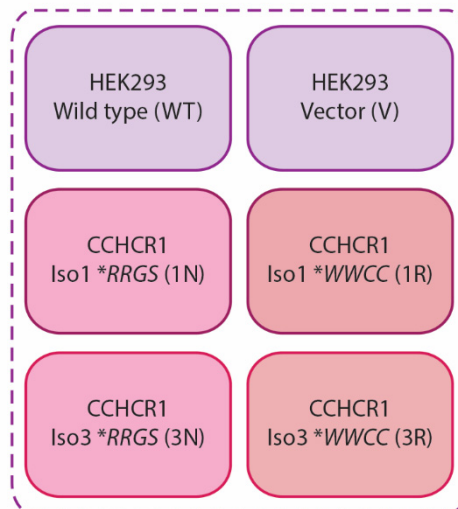


Figure 7 RNA sequencing: CCHCR1 cell lines. CCHCR1 RNA-seq study compared the transcriptomes of the different cell lines overexpressing CCHCR1 with the wild type and vector control cell lines.

MATERIALS AND METHODS

1. Patient material

1.1. Blood samples (III)

We utilized DNA samples from Finnish and Swedish psoriasis families for the association analysis (Inerot et al., 2005; Kainu et al., 2009; Suomela et al., 2007). Thus, we used family trios to investigate association of genetic markers of interest by measuring their transmission from parent to offspring. The Swedish blood samples were collected with the help of the Swedish Psoriasis Association and approved by the Regional Ethics Committee, the Finnish samples were approved by the Ethics Committees of Helsinki, Turku, Tampere, and Oulu University Central Hospitals and Central Hospital of Päijät-Häme. The samples used for the association analysis consisted of trios from 508 psoriasis families in total, including 245 Finnish and 263 Swedish families.

1.2. Split-thickness skin grafts and full-thickness biopsies (I, II, III, IV)

Split-thickness skin grafts (SGs) measuring 5×2 cm were collected with a compressed air-driven dermatome with a fixed thickness setting of 4-6/1000 inches, to obtain a representative sample of epidermis to its full thickness with minimal dermis involvement from the donor site skin. Psoriasis patients were sampled from both the lesional and non-lesional skin and healthy control skin was obtained from reductive mammoplastic or microvascular free flap surgery patients. The samples were immediately immersed in RNA stabilization reagent (RNAlater) to ensure minimal manipulation and gene expression changes and the qualities of the SGs were examined from haematoxylin-eosin-stained paraffin sections; too thin samples that were missing a part of the epidermis (e.g. the basal layer) were discarded from further analysis and not selected for RNA-seq (e.g. sample PN.08). Full-thickness skin samples (3-mm diameter punch biopsies) were collected in order to initiate keratinocyte cultures. All participants provided written informed consent under a protocol adherent to the Helsinki Guidelines and the collection of skin samples was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa and by the Committee of Skin and Allergy Hospital, Helsinki University Central Hospital.

2. SNP genotyping and association analysis (III, IV)

For the association analysis, we genotyped a SNPs rs3130453 (here named as *CCHCR1*Iso1/3*) and rs130076 (*CCHCR1*WWCC/RRGS*) from DNA extracted from the Finnish and Swedish blood samples (III). Genotyping was performed with commercial allelic discrimination assays with pre-designed probes and primers (TaqMan). Association of the SNPs in focus was investigated with transmission disequilibrium test (TDT), by using HaploView for the analysis. We also genotyped these SNPs and determined the *HLA-Cw*06:02* genotype, also with commercial allelic discrimination assays with pre-designed probes and primers (TaqMan) (Nikamo and Ståhle, 2012), from DNA extracted from the SGs (IV). Sample C.05 was not genotyped due to lack of sample for DNA extraction.

3. Cell cultures, transfections, and generation of stable cell lines (I, II, III, IV)

Cells cultured in this thesis were: human embryonic kidney (HEK293), human immortalized keratinocyte (HaCaT), and fibroblast-like (COS-7) cell lines and primary normal human epidermal keratinocytes (NHEK, commercial; KC, primary keratinocytes extracted from full thickness samples).

Plasmid transfections for HEK293, HaCaT, COS-7, and NHEK/KC cells were performed with a nonliposome-based transfection reagent (Fugene HD) (II, III). Constructs used for the transfections were cloned in vectors containing either no tag or pDsRed tag (*CCHCR1* transcripts for isoforms 1 and 3 with **WWCC/*RRGS* haplotypes) or GFP tag (shRNA constructs) (pCMV5, pDsRed-Monomer-N1, and pRNAT-CMV3.2/Neo). Stably overexpressing and silenced cell lines were generated by transfecting either *CCHCR1*-pDsRed (Iso1Non-risk, Iso1Risk, Iso3Non-risk, and Iso3Risk) or shRNA constructs or vector into HEK293 cells (III). We measured the overexpression and selected the cell lines that had the most similar fold changes (of these Iso3Non-risk had the strongest overexpression).

Epidermal keratinocytes (KCs) were isolated from the full thickness skin samples with dispase digestion, which was followed by trypsinization (I, II). KCs were cultured in keratinocyte growth medium with 0.06 mM calcium on cell culture disks coated with collagen I. Samples were collected for RNA-seq from early (passage 1; EKC) and late passages (passage 5 or 6; LKC). KCs were also cultured for confocal microscopy.

The cover slips or cell culture wells were coated with collagen I when transfected, used for immunofluorescent stainings, proliferation assay, or treated with nocodazole.

3.1. Cell treatments (III)

Stably CCHCR1-overexpressing HEK293 cell lines were incubated for 1 h with 1 μ M nocodazole at 37°C, for the disruption of the microtubules. Cell cycle was synchronized by overnight incubation with 0.3 μ M nocodazole. Treatment with EGF was performed on subconfluent cells that were grown in the presence of 20ng/ml or 100ng/ml EGF. After 2, 6, or 18 h the cells were lysed for western blotting or RNA extraction.

4. Antibody stainings (II, III, IV)

All targets for the primary antibodies and stains used in this thesis are listed in Table 2.

4.1. Immunofluorescence microscopy (II, III, IV)

Cells for the immunofluorescence (IF) studies were grown on cover slips with collagen I coating and fixed with methanol or 4% paraformaldehyde-phosphate buffered saline solution, depending on the antibody to be used. After paraformaldehyde fixation cells were permeabilized with 0.1% Triton-X100 in PBS. Indirect immunolabelling was carried out for the following proteins by using commercial antibodies (except for CCHCR1 (Asumalahti et al., 2002)): CCHCR1, γ -tubulin, β -catenin (CTNNB1), phospho- β -catenin (S33/37/T41) (P-CTNNB1), α -tubulin, vimentin (VIM), golgi autoantigen, golgin subfamily a 2 (GM130), KRT17, pan-cytokeratin, actin, complex IV cytochrome c oxidase subunit II (MTCO2), caspase recruitment domain-containing protein 6 (CARD6), EDC4, and decapping enzyme 1 A, s. cerevisiae homolog of (DCP1A). The cells were stained with appropriate antibodies and the nuclei with 4',6-diamidino-2-phenylindole (DAPI) and the pictures were taken with a confocal microscope.

4.2. Immunohistochemistry (II, III)

Formalin fixed paraffin sections (5 μ m) were stained with a peroxidase-based method (ImmPRESS™ Reagent kit) and epitope retrieval was carried out by a heat-mediated method in sodium citrate buffer. The following proteins were targeted in the immunohistochemistry (IHC) studies via indirect antibody

labeling: PYCARD, CARD6, interferon-gamma-inducible protein 16 (IFI16), NLR family pyrin domain-containing 10 (NLRP10), and humanin. Normal rabbit IgG was used as a negative control.

Table 2 Targets of primary antibodies and stains used in this thesis

Antibody	Sample	Method	Used in
Actin-phalloidin	HEK293	IF	III
CARD6	KC, SG	IF, IHC, IEM	II
β -catenin	HEK293	IF	III
P- β -catenin (S33/37/T41)	HEK293	IF	III
CCHCR1	HEK293, HaCaT, COS-7, NHEK/KC, SG	IF, IEM, WB	III, IV
DAPI	HEK293, HaCaT, NHEK/KC	IF	II, III, IV
DCP1A	HEK293	IF	IV
EDC4	HEK293	IF	IV
GAPDH	HEK293	WB	III
GM130	HEK293	IF	III
Humanin	SG	IHC	II
IFI16	SG	IHC	II
Pan-cytokeratin	HEK293	IF, WB	III
KRT17	HEK293	IF, WB	III
MTCO2	KC	IF	II
NLRP10	SG	IHC	II
PI	HEK293	FACS	III
PYCARD (ASC, TSM1)	SG	IHC, IEM	II
STAT3	HEK293	WB	III
P-STAT3(Tyr705)	HEK293	WB	III
P-STAT3(Ser727)	HEK293	WB	III
Ac- STAT3(Lys685)	HEK293	WB	III
α -tubulin	HEK293	IF, WB	III
β -tubulin	HEK293	WB	III
γ -tubulin	HEK293, HaCaT, NHEK	IF	III
Vimentin	HEK293	IF, WB	III

IF, immunofluorescence; IHC, immunohistochemistry; IEM, immunoelectrol microscopy; WB, western blot; FACS, fluorescence-activated cell sorting

4.3. Immunoelectron microscopy (II, III)

Cultured cells and skin biopsies were fixed with 4% paraformaldehyde-PBS solution for the immunoelecton microscopy (IEM) studies. Prior to immersion in 2.3 M sucrose-PBS solution, the cell culture samples were additionally immersed

in 12% gelatin-PBS. The skin biopsies (full-thickness) were frozen in liquid nitrogen, from which thin cryosections were cut with a microtome. CCHCR1 was targeted for detection by antibodies and protein-A gold conjugate in the cultured cells and skin samples. PYCARD and CARD6 were labeled from the skin samples. Labeling was detected with a transmission electron microscope.

4.4. Western blot (II, III)

Cell for western blot (WB) were grown on 6-well plates and homogenized with Laemmli buffer containing 5% β -mercaptoethanol. Western blot analysis was carried out by standard SDS-PAGE and immunostaining protocols, by targeting the following proteins via indirect antibody labeling: actin, CCHCR1, KRT17, pan-cytokeratin, STAT3, P-STAT3(Tyr705), P-STAT3(Ser727), acetyl-STAT3(Lys685), and VIM. Immunostaining with antibodies against α or β -tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to control loading. Signals were detected by enhanced-chemiluminescence (ECL).

5. RT-PCR, quantitative real-time PCR, and RNA sequencing

5.1. RNA extraction (I, II, III, IV)

Total RNA was purified from the cell lines and tissue samples with silica-membrane based kits (RNeasy Plus Mini or miRNeasy Mini), complemented with DNase treatment to avoid DNA contamination. RNA concentrations were measured by spectrophotometric and fluorometric methods and the quality controlled by a nanofluidics device (RNA integrity number for all samples >8). Total RNA was reverse transcribed to cDNA using random hexamer primers.

5.2. Reverse transcription PCR (III)

The expression of *CCHCR1* transcript variants 1 and 3 was analyzed in different tissues and cell lines by standard reverse transcription PCR (RT-PCR). The expressions were studied using variant-specific primers in commercial human multiple tissue cDNA and fetal and tumor sample panels, HaCaT and HEK293 cell lines, and NHEK. *GAPDH* expression was used as a control.

5.3. Quantitative real-time PCR (I, II, III, IV)

Quantitative real-time PCR (qPCR) was applied to measure the expression of target genes in the cell line or tissue samples. We used both commercial pre-

designed primers and probes (TaqMan) or designed specific primers and detected the amplification with a DNA stain-based method (SYBR green). Targets quantified were I) Ribosomal phosphoprotein genes *RPLP13A* and *RPLP0* and *GAPDH* (equal amount of the spike-in RNA mix was added to each cDNA synthesis reaction to control for the PCR reaction), II) *CARD6*, *IFI16*, *IL8*, *PYCARD*, *RPLP13*, and *GAPDH*, III) *CCHCR1*, *KRT17*, *TATA-binding protein (TBP)*, and *GAPDH*, and IV) synaptotagmin 1 (*SYT1*), *IL8*, amphiregulin (*AREG*), talin 1 (*TLN1*), fibronectin 1 (*FN1*), protein phosphatase 2 (*PPP2CA*), and hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT1*).

5.4. RNA sequencing (I, II, III, IV)

Qualified total RNA samples were used for RNA-seq library preparation according to the STRT protocol, which was adjusted for 10 ng samples. The libraries, of 3 technical replicates, were sequenced using an ultra-high-throughput sequencing system (Illumina). Preprocessing of the reads, alignments, and per-gene quantitations were analysed using an established pipeline (Islam et al., 2012). The 5'-end regions of the assembled transcripts were merged as Transcript Far 5'-ends (TFEs), which were annotated with UCSC genes (II, III). Reads aligned within the TFEs were counted by samples again, and normalized using the eight synthetic spike-in RNAs of known concentration that were added to each sample. SAMstr (Katayama et al., 2013) and SAMseq (Li and Tibshirani, 2013) packages were used for the differential expression analysis. Differentially expressed genes or transcripts were extracted by multiclass response test; threshold of significance was Local-FDR < 1% (I) or 5% (II, IV).

6. Cell proliferation and morphology (III)

The morphology of the cells was observed from the confocal microscopy images and the size of the DAPI stained nuclei in the stable cell lines measured from the images. The cell proliferation of stably overexpressing *CCHCR1* cell lines was determined with an automated cell counter. Cells were seeded on 12-well plates and after 24 and 48 h of incubation they were trypsinized and counted. We also utilized DNA-binding dye-based cell proliferation assay (CyQuant) to study cell proliferation. Briefly, the cells were seeded on 96-well plates and allowed to grow for 0–48 h. The cells were frozen at specific timepoints and stained; the fluorescence was measured with a label reader. We used flow cytometry to study cell cycle in the *CCHCR1* cell lines. We synchronized the cells with nocodazole

into G2/M-phase, to determine cell cycle progression of the synchronized cells. The cells were collected at three time points (0, 5, and 10h) and fixed with ice cold 70% ethanol. After RNA depletion with ribonuclease A, the nuclei were stained with propidium iodide (PI).

RESULTS

1. RNA sequencing (I, II, IV)

We collected split-thickness skin graft (SG) biopsies, with minimized inclusion of dermis, from nine control (C), five psoriatic non-lesional (PN), and six lesional (PL) donors and extracted total RNA (Figure 6). Full-thickness skin biopsies were collected from the control donors for keratinocyte (KC) cultures from which total RNA was isolated at early (1st; EKC) and late (5th-6th; LKC) passages. HaCaT cells were cultured and RNA extracted from them as well (I, IV, Figure 7). These samples were used for two separate studies: *keratinocyte* study (C/SG, EKC, LKC, and HaCaT) and *psoriasis* study (C, PN, and PL). Thus, the data from the control (C) samples were used in two different studies and publications.

We also extracted RNA from the CCHCR1 overexpressing cell lines (WT, V, Iso1N, Iso1R, Iso3N, and Iso3R), which were used in the *CCHCR1* study (Figure 7). The study also included CCHCR1 knock down cell lines. The extracted RNA samples were subjected to 5'-end RNA-seq. (IV)

1.1. Varying polyA+ RNA content in different samples (I)

When comparing the expression profiles of samples, a common assumption is that the individual cells contain equal amounts of RNA. Different tissue or cell types, however, might not have equal amounts of RNA per cell. Therefore, we compared the differences in polyA+ RNA contents in our samples. We employed a recently developed method for normalization, SAMstrt (Katayama et al., 2013), which utilizes spike-in RNAs. The estimated polyA+ RNA contents, which were quantified against the added spike-in RNA controls, varied in different sample types (I). This can lead to the misinterpretation of differential expression when traditional endogenous gene-based normalization is applied. Validation by qRT-PCR confirmed the spike-in RNA normalization-based upregulation of two housekeeping genes *RPLP0* and *RPL13A*, both of which were predicted to remain unchanged or downregulated by the gene-based normalization. In conclusion, the use of spike-in-based normalization produced consistent results with qPCR validations, whereas the traditional gene-based normalization method led to inaccurate expression profiles.

2. Characterization of the keratinocyte study samples (I)

We assessed the transcriptome profiles of the healthy skin SGs, KCs (early and late), and HaCaT cells. Hierarchical clustering confirmed significant dissimilarity between the three sample types and we found 11,908 DEGs altogether. The STRT RNA-seq method with synthetic RNA spike-in normalization reflected the activity of the cell type and revealed variation of polyA+ RNA content per total RNA in the different sample types. We used principal component analysis (PCA) (Figure 8a) to elucidate dissimilarity between the samples, and the principal components with gene set enrichment analysis (PC-GSEA) to find the associations between genes and phenotypes. The principal components (PCs) revealed divergence in differentiation and mitochondrial phenotypes between SGs and cultured cells, G1/S-transition between HaCaTs and EKC, and senescence and cellular aging responses between HaCaTs and LKCs. The tissue samples differed from the cultured cells, as expected, and the HaCaT cell line differed remarkably from the other cultured cells, as shown by PCA and by their cytokeratin profiles.

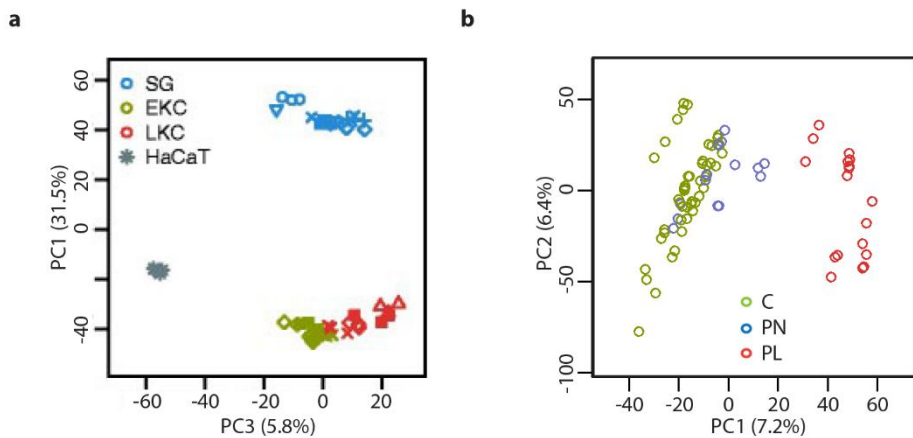


Figure 8 Principal component analysis classification of the samples used in the keratinocyte and psoriasis studies. a) PC1 demonstrates the difference between SGs and other sample types, whereas PC3 separates HaCaTs from KCs and SGs. Symbols in SGs and KCs illustrate identical donors in three technical replicas each. SG, Split-thickness skin graft; EKC, early passage keratinocyte; LKC, late passage keratinocyte. b) PC1 illustrates the clustering of healthy control (C), psoriasis non-lesional (PN) and lesional (PL) samples. Percentages beside of the axis labels are the contribution ratios. Modified from I and II.

3. Characterization of the psoriasis study samples (II)

In the psoriasis study we investigated the clustering of SG samples by PCA (Figure 8b), which revealed clustering of the three different sample groups but significant overlap of the non-lesional and healthy control skin samples (PN and C) and separation from the lesional samples. Some non-lesional samples clustered between the control and lesional samples, suggesting transcriptional alterations already in the non-lesional skin.

We performed group-wise (GW) comparisons between the three sample groups at first. The comparison of lesional sample group with the control (PLvsC) or non-lesion (PLvsPN) groups revealed 2436 and 3541 upregulated and 2550 and 494 downregulated transcripts, respectively (Fold Change (FC) >1.5 and <0.75, False Discovery Rate (FDR) <0.05). According to positional analysis: the upregulated transcripts showed enrichment from *PSORS4* locus. The GW comparison of non-lesion with control skin (PNvsC) identified 35 DETs; 28 of which were upregulated and 7 downregulated. Interestingly, 12 of the transcripts mapped to the known PSORS loci; *PSORS4* was the most represented among the upregulated transcripts, as shown also in previous studies (Gudjonsson et al., 2009). Of the differentially expressed transcripts, we selected the classes that represented annotated genes and identified 2720 (PLvsC), 2610 (PLvsPN), and 25 (PNvsC) DEGs. We also compared the expressions pair-wisely between lesional and non-lesional skin from each psoriatic patient separately (data not shown) to see if the different approach in analysis would alter the results from pathways analysis or if the medications had significant effects. We analyzed the DEGs that were shared in all patients and got similar results as from the GW analysis.

4. Expression profiling (II)

4.1. Psoriasis non-lesional skin

The comparison of the two healthy skin sample groups, psoriasis non-lesional with the control samples, revealed upregulation of genes for keratinocyte and epidermal differentiation and defense response already in the non-lesional samples. Most of the upregulated transcripts were induced also in the lesions (PLvsC) and highlighted the EDC region (*S100A7*, *S100A12*, *SPRR2A*, *SPRR2B*, *SPRR2D*, *SPRR2G*, and *LCE3E*). There were two unique transcripts, however; contactin-associated protein-like 3 (*CNTNAP3B*) and the mitochondrial transcripts (ChrM) named in the alignment step as TVAS5, both of which have

not been implicated in psoriasis before. The most frequent mitochondrial reads mapped at the start site of mitochondrially encoded 16S ribosomal RNA (*MTRNR2* gene) that encodes for a polypeptide called humanin.

Among the downregulated transcripts in the non-lesional samples we identified only three DEGs one of which, interestingly, was the nuclear gene homolog of *MTRNR2*: *MTRNR2L1* (humanin-like). Due to the high similarity in sequence among humanin-like genes (Bodzioch 2009), the specific quantitation of humanin and its nuclear homologs was challenging. We demonstrated that humanin and humanin-like proteins are strongly expressed in keratinocytes but were unable to detect any difference between the three sample types. As the RNA-seq data exhibited disturbed gene expression in the non-lesional skin, it remains to be studied whether humanin and its homologs play a role in the pathogenesis of psoriasis.

4.2. Psoriasis lesional skin

We investigated the DEGs from the PLvsPN and PLvsC comparisons (group-wise, GW) with pathway and functional analysis and got similar results from both of the comparisons; thus, many similar pathways and functions were highlighted in both comparisons. Therefore, we analyzed the DEGs that are shared in the two comparisons. Functional annotation analysis highlighted enrichment of the upregulated genes in epidermal differentiation-related gene ontology (GO) groups that included the EDC region encoded genes (*LCE* and *SPRR*). Defense response, oxidoreductase, protease, and lipid degradation were among the most significant functional clusters as well. Caspase recruitment domain (CARD) and caspase gene families were highlighted in the analyses. Pathway analyses identified enrichment in e.g. lysosome, NOD-like receptor (NLR), and RIG-I-like receptor (RLR) signaling pathways. Missing from the most significant and largest groups in the GW-PLvsC comparison; the analysis of the upregulated genes from the GW-PLvsPN comparison highlighted GOs related to mitochondria and oxidative phosphorylation, showing enrichment also in the pair-wise comparison. The absence might, however, result from the heterogeneity of the patients.

We focused next on the NLR signaling pathway, which was highlighted as a upregulated pathway in the lesional samples. RLR signaling and cytosolic DNA sensing pathways rose up as well and all three pathways shared several genes. The NLR signaling pathway included several highly upregulated transcripts:

nucleotide-binding oligomerization domain protein 2 (*NOD2*), *CARD6*, *CARD18*, *CASP5*, *IL1B*, *IL8*, and chemokine *CXCL1* (GW-PLvsPN, FC >1 x 10⁸). Also several other NLR signaling-related components, with less upregulation, were identifiable: *NLRP10*, NLR family member X1 (*NLRX1*), *CASP1*, *CASP8*, and *PYCARD* (ASC). The receptors of the cytosolic DNA sensing and RLR signaling pathways; DNA-binding receptor genes *AIM2* and *IFI16* and RNA helicase protein genes *IFIH1* and *DDX58* (RIG-I), were also upregulated. Several other RLR-related transcripts were upregulated as well, including ubiquitin-like modifier *ISG15* and *CYLD*.

We verified the upregulation of *CARD6*, *IFI16*, *PYCARD*, and *IL8* in lesional skin samples by qPCR. In addition, we selected a few proteins, encoded by the DEGs *NOD2*, *PYCARD*, *IFI16*, *CARD6*, and *NLRP10*, whose expression pattern has not been thoroughly studied in psoriatic skin before, or it has remained unclear. We used immunohistochemistry to examine and verify the expression and localization of the proteins. Immunohistochemistry demonstrated that *NOD2* expression, indeed, was induced in the lesional epidermis, including keratinocytes. The expression varied between individuals in psoriasis non-lesional and lesional skin and in the non-lesional samples, especially, there was more variation from weak to increased expression. On the cellular level, *NOD2* was localized in the cytoplasm and in some cells on the cell membrane. *PYCARD* expression in the epidermis was observed in all sample groups. The expression level and pattern, however, differed in the lesions, where the expression was strongly induced in the cytoplasm, and in some cells in the nucleus. The overall *PYCARD* staining in the non-lesional samples was weaker and some samples showed nuclear staining. The control skin exhibited only a few *PYCARD* positive nuclei, and its overall staining was weaker than in the psoriasis patients. The cytoplasmic *PYCARD* induction in the lesional samples was observable also in IEM. Interestingly, in some keratinocytes the *PYCARD* labeling formed clusters (diameter around 500 nm) that localized with cytoplasmic membrane structures, possibly small vesicles. *IFI16* staining was localized into cell nuclei in the psoriasis samples and strongly upregulated especially in the lesional epidermis. Controls had only a few *IFI16* positive nuclei and in some samples we detected weak cytoplasmic expression, which was absent from the psoriatic SGs.

CARD6 protein was detectable as granular cytoplasmic staining and also in nuclei. We identified the granular staining as mitochondria, by colocalizing with

mitochondrial marker MTCO2. The localization was verified also by IEM, in which the CARD6 was observed at the cell-cell contacts as well. Some of the non-lesional samples showed induced expression as well but the control skins were almost CARD6 negative. NLRP10 staining was observable all over the epidermis but of the selected NLR signaling pathway proteins, it remained as the only one for which we couldn't detect any difference between psoriatics and controls.

The shared genes of the GW-PLvsPN and -PLvsC comparisons contained 220 downregulated genes that were enriched in such functions as: extracellular matrix, blood vessel development, and cell junction. Pathway analysis recognized, e.g., pathways in cancer, cytokine-cytokine receptor interaction, and focal adhesion. The comparison PLvsC recognized several DEGs that were absent from the PLvsPN comparison; the separate analysis therefore revealed pathways that were unidentified in the PLvsPN comparison, such as Wnt, TGF- β , and Notch signaling.

5. RNA-seq of skin graft samples refined previous findings in psoriasis (II)

We wanted to estimate the advantages of the methods used in this study design by comparing our RNA-seq data of SG samples with: two large microarray studies of full-thickness samples by Gudjonsson et al. and Tian et al. (Gudjonsson et al., 2009; Tian et al., 2012) and one with microdissected epidermis and dermis by Mitsui et al. (Mitsui et al., 2012) and with a RNA-seq study of full-thickness samples by Li et al. (Li et al., 2014). The comparison with two microarray studies done on full-thickness samples of psoriatic lesional and non-lesional skin resulted in the detection of 2232 DEGs that were unique only in our study. The RNA-seq of psoriatic full-thickness lesional and control skin differed from our study in several ways but similar functional categories and pathways were identifiable in both studies. The studies shared 1566 DEGs but numerous unique transcripts as well; 1200 and 7515 DEGs in the SG or full-thickness skin, respectively. Genes that were unique for our SG samples were enriched in such categories as Wnt signaling, ubiquitin proteasome pathway, lysosome, and focal adhesion. The NLR signaling pathway included DEGs, in our SG samples, which were not recognized from the full-thickness samples (e.g. *CASP1*, *CASP8*, *CARD18*, *CYLD*, and *TNFAIP3*). DEGs enriched in lymphocyte (upregulated), muscle, or secretion (downregulated) were recognized in the full thickness samples whereas in our SGs they were missing or not among the significantly altered ones.

The microdissected epidermis and dermis samples were from lesional and non-lesional psoriatic skin. When we compared their DEGs with our data, 517 were shared and 2339 unique for our SGs and 679 for the microdissected epidermis. We identified, for example, several *LCE*, *SPRR*, and *KRT* genes that were undetectable in the microarray. Among NLR signaling; genes such as *NOD2*, *PYCARD*, *DDX58*, *CASP1*, and *IL8* were recognized in both studies but transcripts for *CARD6*, *CARD18*, *CASP8*, *IL1B*, and pyrin domain-containing protein 1 (*PYDC1*) were upregulated only in our study. Only 3% of the DEGs from our study were detectable in the microdissected dermis data. To validate whether the variation on the amount of dermis between the skin samples has an effect on our RNA-seq results, we compared the expression of fibroblast specific genes, *COL3A1* and *COL1A2*, in three sample groups. Some of the samples exhibited a slight decrease in the expression of these markers, suggesting that the architecture and thickening of the epidermis in lesional samples can create some downregulation of the dermal components. When compared with the non-lesional samples; the relative decrease of the dermis in the lesional samples is more pronounced in SGs than in full-thickness samples. The number of downregulated genes in the lesional SG samples, however, is lower than the number observed in the full-thickness studies.

6. Functional characterization of the psoriasis candidate gene *CCHCR1*

6.1. Association of a SNP within *CCHCR1*, with psoriasis (III, IV)

We genotyped the SNP rs3130453 (G/A) in 508 Finnish and Swedish psoriasis families (III). The A allele, that encodes for a stop codon, thus enabling the translation of only *CCHCR1* isoform 3 (named here as **Iso3* allele), showed preferential transmission from heterozygous parents to affected offspring ($P < 10^{-7}$). We also genotyped, from the same family material, the SNP rs130076 (C/T) from in the *CCHCR1*WWCC* ("Risk") haplotype. The risk allele (T) showed association with psoriasis ($P < 10^{-13}$), as expected. We extended, therefore, the risk allele as *CCHCR1*Iso3WWCC* for the haplotype analysis showed the transmission of **Iso3Risk* ($P < 10^{-16}$) to affected offspring. We also analyzed these SNPs from the SG samples (IV). There were thus eight controls and seven psoriasis samples. Five of the psoriatic samples were homozygous for the *CCHCR1*Iso3* allele, whereas only two out of eight were homozygous in the controls. None of the psoriatic samples had the homozygous **Iso1* genotype. Five out of seven psoriatic samples were heterozygous for the Risk (**WWCC*) haplotype whereas five out of eight controls had the homozygous Non-risk haplotype. One of the control samples, however,

had the homozygous Risk haplotype. We also genotyped the most known psoriasis-associated haplotype *HLA-Cw*06:02* and found that most of our control samples were negative for the haplotype (six out of eight) but most of the psoriasis samples were heterozygous positive (five out of seven).

Table 3 CCHCR1*Iso3WWCC and HLA-Cw*06:02 genotypes of the SG samples

Sample	<i>CCHCR1*Iso3</i>	<i>CCHCR1*WWCC</i>	<i>HLA-Cw*06:02</i>
C.02	NP	NP	NN
C.04	NN	NN	NN
C.07	NP	NN	NN
C.09	PP	NP	NP
C.10	NP	NN	NN
C.11	NN	NN	NN
C.12	PP	PP	NP
C.14	NP	NN	NN
P.02	PP	NP	NP
P.03	NP	NP	NP
P.05	PP	NP	NP
P.06	PP	NP	NP
P.07	PP	NP	NP
P.08	PP	NN	NN
P.09	NP	NN	NN

NN= homozygote (negative), NP= heterozygote, PP= homozygote (positive)

6.2. Localization of CCHCR1 at the centrosome and P-bodies (III, IV)

We localized both the endogenous and overexpressed CCHCR1 at the centrosome in our studies (III, Figure 9). The endogenous CCHCR1 was investigated in HEK293 and HaCaT cell lines and transiently transfected CCHCR1 was studied in NHEKs. Most of the functional studies, however, were performed in the stably overexpressing HEK293 cell lines, in which both CCHCR1 isoforms, with either Risk or Non-risk haplotype, showed overlapping or adjacent expression with the centrosomal marker γ -tubulin. In the centrosome, CCHCR1 also colocalized with β -catenin and its phosphorylated form. IEM studies with the stable HEK293 cells overexpressing isoform 1 revealed that CCHCR1 is present at the pericentrosomal region. The overexpressed CCHCR1 was detectable throughout the cell cycle. The localization, however, was dynamic and fluctuated especially during mitosis; CCHCR1 localized at the midbody near the end of the cytokinesis. (III)

CCHCR1 was also visible as cytoplasmic granules, the size of which varied between the overexpressed isoforms (III). Coiled-coil region-containing proteins

form aggregates easily. Likewise CCHCR1, the majority (75%) of centrosomal proteins contain coiled-coil regions in their structure (Andersen et al., 2003). We noticed, however, that Iso3Risk formed larger granules in the cytoplasm, when compared with the other three forms (both in the stable cell lines and with transient transfections, in different cell lines). As CCHCR1 Iso3Non-risk was recently localized at P-bodies (Ling et al., 2014), we studied if the overexpressed CCHCR1 colocalizes with the P-bodies, by immunostaining the P-body markers EDC4 and DCP1A (IV). Some of the granules colocalized with the P-bodies, in the stable cell lines, but we noticed a difference between the isoforms; in the isoform 1 -overexpressing cell lines (especially Iso1Non-risk), the P-body markers had only seldom colocalization with CCHCR1. In the isoform 3 -overexpressing cell lines, however, the P-body staining overlapped almost completely with the cytoplasmic granules, including the centrosomal CCHCR1. Immunofluorescent staining of vimentin suggested, however, that the centrosomal CCHCR1 was not aggresomal accumulation, which is an organelle composed of misfolded aggregated proteins, surrounded by a vimentin cage, and located adjacent to the centrosome (I) (Johnston et al., 1998). We also excluded the possible localization of Iso3Risk CCHCR1 with the cis-golgi, as it surrounds the centrosome and the showed strong perinuclear staining, especially in transiently transfected NHEK cells (III).

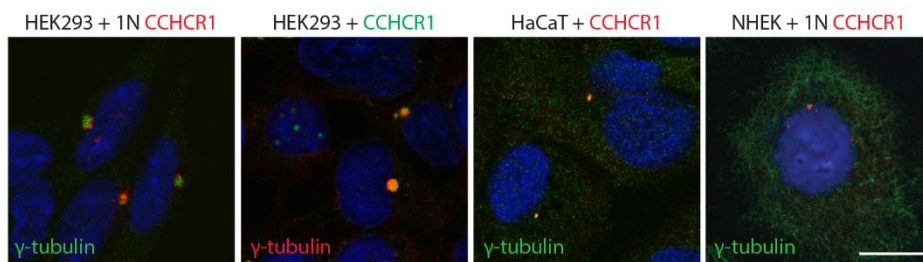


Figure 9 Localization of CCHCR1 at the centrosome. The first figure illustrates the adjacent localization of Iso1Non-risk CCHCR1 (stable cell line) with the C-terminal pDsRed tag to the centrosomal marker γ -tubulin. The next two figures illustrate the colocalization of the endogenous CCHCR1 (stained with an antibody tagging the N-terminus) with the γ -tubulin. Nuclear staining is also observable in the HEK293 figure. The last figure shows the localization of CCHCR1, in transiently transfected NHEK cells, at the centrosome. Scale bar: 10 μ m. Modified from III.

Despite the absence of DsRed-tagged CCHCR1 isoforms (tag in the C-terminus) in the following locations, the endogenous protein (stained with an antibody

against the N-terminal part of isoform 3) was detectable also at the cell-cell borders and spots in the nucleus (III). IEM revealed labeling in the close proximity of cell membranes in association with desmosomes both in psoriatic and healthy skin samples. These additional locations suggested that the C-terminus was modified or cleaved. Western blot supported the modification: an additional band with smaller size was seen under the full-length sized CCHCR1 band.

6.3. CCHCR1 affects cytoskeleton, cell morphology, and cell cycle (III, IV)

The stable overexpression of CCHCR1 affected the morphology of the HEK293 cells; isoforms 1 and 3 having opposing effects on the size and shape. Iso1Non-risk CCHCR1 affected the cell size and shape to appear bigger and rounder, than the other cell lines. Both isoform 3-expressing cell lines appeared smaller than the isoform 1-expressing cells and had more membrane protrusions and also smaller nuclei in interphase ($P < 10^{-6}$). The Iso1Non-risk cell line, especially, also exhibited multilobular nuclei, suggesting aberrations in cell division. (III)

We also studied the relationship between CCHCR1 and the cytoskeleton (III). We focused on the microtubulus network, as its organization is regulated by the centrosome, alongside with actin, vimentin, and cytokeratins. We used nocodazole to disrupt the microtubule structures in the overexpressing cell lines, which increased the number of cytoplasmic CCHCR1 granules. CCHCR1 was observable in the centrosome as well, suggesting that the localization was partially dependent on the microtubules. The disruption also affected the attachment and shape of the Iso3Risk cells, which clumped together. The isoform 3 -overexpressing cells exhibited also abnormalities in the actin cytoskeleton, especially after the disruption of the microtubules: the actin forms punctate staining in the cytoplasm. Vimentin intermediate filaments were only slightly altered and lacked similar alterations in organization as actin, after the nocodazole treatment

IF and WB showed downregulation of cytokeratin expression especially in the Iso3Risk cells. Downregulation of the cytokeratins was observed also in the CCHCR1-silenced HEK293 cell lines. We focused on a specific cytokeratin, KRT17, which in the overexpressing CCHCR1 cell lines revealed increased expression by IF in Iso1Non-risk cells. The isoform 3 cells, however, showed expression only in a few cells. The expression levels were verified with WB and qPCR. Furthermore, the silencing of CCHCR1 downregulated the expression of KRT17. Stimulation

with EGF induced the expression in all the other cell lines, except in the isoform 3-overexpressing and silenced ones.

As the morphology of the different CCHCR1 overexpressing cell lines already suggested, the RNA-seq of the CCHCR1 overexpressing cell lines confirmed that there were changes in gene expressions related to cell adhesion; downregulated genes of the Iso1Risk and the isoform 3-overexpressing cell lines were enriched for example in focal adhesion pathway.

CCHCR1 isoform 3-overexpressing cells differed from the other cell lines by proliferating more vigorously; we counted the cell number with an automated cell counter after growth period of 1 or 2 days and determined that the cell number was 40–60% higher. We measured the cell proliferation also with a DNA stain-based cell proliferation assay that, however, did not reveal any statistically significant differences. Differences in the size of nuclei may have an effect on cell proliferation methods based on DNA staining. We measured the cell cycle profiles of the overexpressing cell lines with FACS analysis. The analysis results lacked evidence of the effects of CCHCR1 overexpression on the cell cycle, except for apoptosis, which was significantly higher in the Iso3Risk cells, especially after synchronization by microtubules network disruption ($P < 0.03$). Transcriptome profiling with RNA-seq, however, identified upregulation of cell cycle-related pathways especially in the Iso1Non-risk overexpressing cell line, which exhibited signs of disturbed cell division in the form of multinucleated nuclei. Proliferation and cell cycle analysis of CCHCR1-silenced shRNA-cell lines was also measured but lacked significant effects.

6.4. CCHCR1 regulates EGF-induced STAT3 phosphorylation (III)

We investigated the effect of epidermal growth factor (EGF) stimulation on the HEK293 cells with stable CCHCR1 overexpression or downregulation. The stimulation induced the CCHCR1 overexpression even further and affected its localization; CCHCR1 was still present at the centrosome but its cytoplasmic localization increased. The increase in expression was detected both on RNA and protein level (III). Both of the CCHCR1 isoforms 1 and 3, with Non-risk or Risk haplotype, responded to the EGF treatment. As EGF activates STAT3, we studied also the effects of CCHCR1 isoforms on the phosphorylation of STAT3 by immunoblotting, using antibodies against tyrosine 705 or serine 727 phosphorylated and lysine 685 acetylated STAT3. The isoform 1 overexpression

induced the STAT3 tyrosine 705 phosphorylation, whereas isoform 3 lacked the same effect. The effect was induced even further when stimulated with EGF. In addition, the silencing of CCHCR1 decreased the activation. Expression level of STAT3 and its serine 727 phosphorylation or lysine 685 acetylation remained unaffected.

6.5. CCHCR1 affects the expressions profile of cultured cells with haplotypic effects (IV)

We assessed gene expression profiles of the CCHCR1 overexpressing HEK293 cell lines by RNA-seq. All the four different forms (Iso1Non-risk, Iso1Risk, Iso3Non-risk, and Iso3Risk) were compared with the expression levels in the controls, including wild type HEK293 (WT) and Vector (V). Several genes were upregulated and downregulated and the different forms had an effect on many different genes but also all shared 209 upregulated ($FC > 1.5$) and 618 downregulated ($FC < 0.75$) genes ($FDR < 0.25$). We used the DEGs for functional and pathway analysis. Upregulated functions included: regulation of transcription (genes containing zinc finger domains), and protein phosphorylation by serine/threonine kinases. Interestingly, negative regulation of transcription from RNA polymerase II promoter was among the functions as well. Downregulated genes also enriched in functional categories related to transcriptional regulation but also blood vessel morphogenesis and genes with EGF calcium binding domain were highlighted. The downregulated genes were also enriched in calcium signaling and regulation of actin cytoskeleton pathways.

7. RNA-seq exhibits similar pathways and functions in psoriatic skin and in cells with disturbed gene expression by CCHCR1-manipulation (IV)

We compared the expression profiles of the psoriatic SGs and the CCHCR1-overexpressing or silenced cell lines. The two different study groups shared functions and pathways. Notably, as we described in the psoriasis study (II), the NLR and RLR signaling pathways were upregulated. In the CCHCR1-overexpressing cell lines, however, the pathways were downregulated. We compared all the DEGs from the overexpressing cell lines with the DEGs from the psoriasis SGs. The two groups shared 1962 DEGs that in the GO analysis were enriched in functions and pathways related to e.g. metabolism of proteins, signaling by Wnt, mitochondria, gene expression, cell cycle, and infections.

Interestingly, humanin-like genes, encoding an anti-apoptotic peptide that was mentioned in the RNA-seq of non-lesional psoriatic skin, were among the DEGs detected by RNA-seq. The RNA-seq of overexpressing cell lines identified several other nuclear homologs of *MTRNR2*, of which *MTRNR2L7* was highly downregulated in Iso1Risk and in both isoform 3 -overexpressing cells.

Table 4 Main results of CCHCR1 functions studied in this thesis

Function	Effect
Regulation of cytoskeleton	Alterations in the cell morphology
Cytokeratin	Iso1N upregulates expression; especially KRT17, which is upregulated also by iso1R. Iso3 and CCHCR1 silencing inhibits expression
Actin	Isoform-specific effect of CCHCR1 on the actin cytoskeleton and the overexpression affects the regulation of actin cytoskeleton pathway. Actin forms punctate staining in iso3R after disruption of the microtubulus cytoskeleton
Vimentin	Downregulated especially in the iso3 -overexpressing cells
Size of the nucleus	Biggest in iso1N, whereas smaller in iso3 cells. Iso1N exhibits also increased number of large cells with multilobulated nuclei
Proliferation	Iso3 cells multiply faster than Iso1 cells or controls
Cell death	Iso3R cells exhibit larger population of dying, possibly apoptotic, cells
STAT3 phosphorylation	P-Tyr upregulated in iso1 cells, iso3 and downregulation of CCHCR1 inhibits phosphorylation even when stimulated by EGF
Localization of P-bodies	P-bodies colocalize rarely with iso1, whereas iso3 colocalize with P-bodies, also at the centrosome

1/iso1; isoform 1, 3/iso3; isoform 3, N; non-risk, R; risk, P-Tyr; tyrosine phosphorylation

DISCUSSION

1. STRT RNA sequencing with spike-in normalization

The STRT RNA-seq method, complemented with the synthetic spike-in RNAs, revealed variation in polyA⁺ RNA content per total RNA in different cell types; skin grafts of normal and psoriatic skin and cultured keratinocytes and HaCaT cells. Even though the STRT reads arise from the ultimate 5' end of the polyA⁺ transcripts and the method lacks information on the splice variants, the advantages include especially the identification of transcription start sites and strand information (Islam et al., 2012; Swindell et al., 2014). The small amount of starting material needed for library preparation, early multiplexing, and the inclusion of external spike-in RNAs as a standard procedure can also be included as advantages. The spike-in normalization has been shown to be a valuable tool when comparing samples with fluctuating polyA⁺ RNA contents (Katayama et al., 2013; Loven et al., 2012) and we showed that it produced consistent results with qPCR validations. KCs have been shown to change their RNA content over time in long-term culture (Staiano-Coico et al., 1986) and here we showed that the tissue samples differed from the cultured cells. The approach is applicable also for other studies with fluctuating polyA⁺ RNA content, here it was suitable for the transcriptome study on the CCHCR1-overexpressing cell lines that also had variation on their cell size and thus total RNA content.

2. Transcriptional profiling of psoriatic epidermis

Several transcriptomics studies have been conducted on psoriatic skin, most of them using full-thickness biopsies. Our approach was to focus on the epidermis. The results highlighted alterations in functions related to epidermal homeostasis in the psoriatic lesional skin and already in the non-lesional skin. The most notable difference between the full-thickness and skin graft transcriptomes of psoriasis was that lymphocyte, muscle contraction, and secretion were highlighted only in the full-thickness samples that contain large amount of dermis. Furthermore, it has been suggested that dermis-derived transcripts are driven downward by the expansion of epidermis in psoriatic lesions (Li et al., 2014) or by gene length bias (Swindell et al., 2014), both of which we took into consideration by using the skin graft samples and by the 5' end targeted RNA-seq method.

There are only a few transcriptional studies conducted with psoriatic non-lesional skin compared with normal skin, this one being the first that focuses on the epidermal transcription. We identified 35 differentially expressed transcripts, many of which (12 DETs) mapped to the known psoriasis susceptibility loci. *PSORS4* was the most represented and has been already discovered by previous studies (Itoh 2005, Gudjonsson 2009). Most of the upregulated genes in the non-lesional skin were upregulated also in the lesional samples, except for *CNTNAP3B*, and the mitochondrial transcripts that mostly represent *MTRNR2*. Among the downregulated genes, we identified *MTRNR2L1* (humanin-like), which is the nuclear gene homolog of *MTRNR2* (humanin). The specific quantitation of humanin and its homolog was difficult due to high sequence similarity. However, we demonstrated that humanin was strongly expressed both in psoriatic and healthy skin. Humanin and its homologs are peptides with antiapoptotic and cytoprotective activities (Yen et al., 2013; Zapala et al., 2011) but have not been implicated in psoriasis or in skin before. Its receptor agonist *CALML5* (Hashimoto et al., 2013; Mehul et al., 2006), however, has been linked with psoriasis. The role of humanin in the pathogenesis of psoriasis remains to be studied.

The main advantage of RNA-seq in the transcriptome studies is the increased sensitivity, which enabled us to identify more DEGs than the previous microarray studies. Innate immunity, including NLR signaling and inflammasome activation, were highlighted in the analysis from lesional skin. It has been previously shown that the key components of the inflammasome are present in keratinocytes (Watanabe et al., 2008) and we investigated further the expression of *NOD2*, *PYCARD*, *IFI16*, *CARD6*, and *NLRP10* to validate the possible activation of these innate immunity-related pathways on protein level. Except for *NLRP10*, previous transcriptome studies (Li et al., 2014; Mitsui et al., 2012) have identified their induction in lesional skin. *NOD2*, a key component of the NLR signaling pathway, exhibited induced expression in psoriatic epidermis, especially in keratinocytes. Several alleles of *NOD2* have been associated with inflammatory diseases, including atopic eczema and arthritis (Zhong et al., 2013). Their role in psoriasis, however, has not been confirmed (Zhu et al., 2012a). NLRs, such as *NOD2*, *NLRP10*, and *NLRX1*, which were upregulated in our lesional samples, operate via *RIPK2* by regulating the nuclear factor- κ B and mitogen-activated protein kinase pathways that lead to the production of chemokines, cytokines, and antimicrobial peptides (Latz et al., 2013; Zhong et al., 2013). Though highly upregulated in lesional skin, the gene *CARD6* has not been genetically associated

with psoriasis. Its function is unclear; it may play a role in immune defense via interaction with RIPK2 (Dufner et al., 2008). We detected CARD6 in the cytoplasm but also in the mitochondria, which is a novel localization for the protein. Interestingly, a small fraction of NOD2 is also localized, with another inflammasome-related protein; mitochondrial antiviral-signaling protein (MAVS), at the mitochondria (Sabbah et al., 2009). Some drugs used for the treatment of psoriasis, such as dithranol, function via the mitochondria. The number of mitochondria is increased in the psoriatic epidermis and their function altered via changes in the cristae when treated with dithranol (Swanbeck and Lundquist, 1972). The shape of the mitochondrial cristae determines the respiratory efficiency and effects on the cell growth via the assembly of the respiratory chain supercomplex assembly (Cogliati et al., 2013).

PYCARD, a key component of the inflammasomes, was upregulated also on protein level in our lesional samples. Its location was predominantly cytoplasmic, but in some cells also nuclear. Closer investigation of the keratinocytes in the lesional samples revealed cytoplasmic PYCARD clusters that may represent inflammasomes. Some of the clusters were associated with membrane structures. Interestingly, lysosome was most enriched in our pathway analysis and it has been shown that lysosome rupture plays a role in NLRP3 inflammasome signaling (Jin and Flavell, 2010). In addition, inflammasomes colocalize with autophagosomes (Shi et al., 2012). It remains to be studied whether the membrane structures in lesional keratinocytes represent lysosomes, autophagosomes, or other vacuoles. The genes for the most studied inflammasome receptors, *NLRP1* and *NLRP3*, were not among the DEGs detected in this study. However, they have been associated with psoriasis (Carlstrom et al., 2012; Ekman et al., 2014). *NLRP10* was a novel DEG discovered in our study and we demonstrated that NLRP10 protein is strongly expressed both in healthy as well as in psoriatic skin. Interestingly, NLRP10 has been suggested as an inhibitor of the inflammasome assembly (Damm et al., 2013) but essential for the initiation of the adaptive immunity by dendritic cells (Eisenbarth et al., 2012) and harbors polymorphisms that are associated with atopic dermatitis but not with psoriasis (Hirota et al., 2012; Zhong et al., 2013).

IFI16 was strongly increased in the lesions, and predominantly present in nuclei. IFI16 and AIM2, which both were upregulated in the lesional samples, encode for DNA-sensing receptors that form an inflammasome with PYCARD (Chiliveru et

al., 2014; de Koning et al., 2012a). There is abundant cytoplasmic DNA in keratinocytes of psoriatic lesions, and thus DNA-sensing receptors have been suggested to play a role in the pathogenesis of psoriasis (Lande et al., 2007; Prens et al., 2008). AIM2 is among the most highly upregulated PRRs in lesional skin (Prens et al., 2008), which agrees with previous findings that illustrate activation of PYCARD by IFI16 already in the cell nucleus (Chiliveru et al., 2014; de Koning et al., 2012a). It has been suggested that in psoriatic lesions IFI16 translocates from cell nuclei into the cytoplasm in a subpopulation of keratinocytes, whereas in non-lesional skin it stays in nuclei (Chiliveru et al., 2014). Our results, however, did not support the translocation of IFI16 to the cytoplasm and the most obvious difference was strong nuclear staining in lesions, but weaker expression in controls. In addition, RNA-sensing receptors *IFIH1* and *DDX58* (RIGI) were induced in the lesional samples and are well known susceptibility genes in psoriasis (Oudot et al., 2009). *DDX58* operates via inflammasome and there is interplay between the RLR and NLR pathways for *DDX58* and *NOD2* regulate each other (Abbott et al., 2007). Our analysis identified also *CYLD*, a psoriasis candidate gene that functions in both pathways (Abbott et al., 2007; Friedman et al., 2008; Oudot et al., 2009).

3. Function of *CCHCR1* and relevance in psoriasis

In the psoriasis susceptibility gene study, we cloned a novel longer isoform 1 encoding transcript. It is transcribed from an alternative TSS (exon 1b) and the SNP (rs3130453) in the following exon 2 determines whether the isoform 1 translated; a stop codon (**Iso3*) inhibits translation and tryptophan (**Iso1*) enables it. The association analyses of psoriasis samples suggested that the **Iso3* allele, which inhibits the translation of the *CCHCR1* isoform 1, associates with psoriasis, along with previously studied risk allele **WWCC.HLA-Cw6*, the main marker of *PSORS1*, is strongly associated with psoriasis. The mechanistic support for its role in this disease is missing and since the whole region is in strong linkage disequilibrium, the investigation of the function and association of other genes, such as *CCHCR1*, is justified. The role of *CCHCR1* as a susceptibility gene for psoriasis has been strengthened by genome-wide association studies where the SNPs investigated also in this thesis (rs130076 and rs3130453) have shown strong association with psoriasis (Liu et al., 2008; Riveira-Munoz et al., 2011; Zhang et al., 2009; Zheng et al., 2011).

The exact cellular localization of CCHCR1 has been undefined, for the expression level of the endogenous protein in cells is extremely low and therefore difficult to detect. We investigated the localization of CCHCR1 and detected it at the centrosome and pericentrosomal region (Figure 10). This novel localization is supported by previous mass spectrometry studies, where CCHCR1 was detected from extracted centrosomes (Andersen et al., 2003; Jakobsen et al., 2011), and later by an immunoprecipitation study where they replicated our pericentrosomal localization result in stably overexpressing CHO-K1 cells (Ling et al., 2014). We also ensured that the pericentrosomal localization was not merely an aggresome, which forms next to the centrosome. This finding was also replicated by Ling et al. (Ling et al., 2014). Furthermore, we reported that CCHCR1 colocalizes at the centrosome with a phosphorylated form of β -catenin, a protein involved in Wnt pathway and implicated in psoriasis (Gudjonsson et al., 2010b). β -catenin has a function in cell-cell adhesion and regulates centrosome splitting and microtubule re-growth the centrosome (Bahmanyar et al., 2008; Huang et al., 2007; Kaplan et al., 2004).

The centrosome plays an important role in cell division, therefore linking the function of CCHCR1 to this event. The localization of CCHCR1 fluctuated during the cell cycle and was observable also at the midbody during cytokinesis. Various centrosomal proteins, including γ -tubulin and β -catenin, have a function in cytokinesis as well and are identifiable at the midbody (Glotzer, 2005; Kaplan et al., 2004; Steigemann and Gerlich, 2009). The formation of multilobulated nuclei in the CCHCR1 overexpressing cell lines, especially in the CCHCR1-Iso1Non-risk cells, also suggests a role in cell division and cytokinesis. The RNA-seq study highlighted *SYT1*, which was highly upregulated in the isoform 3 -overexpressing cells. Interestingly, *Syt1* has been suggested to associate with the MTOC and plays a role in spindle organization in mouse oocytes (Zhu et al., 2012b). IEM of the skin samples also revealed that the CCHCR1 is expressed at the proximity of cell membrane and desmosomes, which was also observed by IF staining of the endogenous CCHCR1 in the cell cultures. Furthermore, it has been shown that during epidermal differentiation the desmosomes replace centrosomes and become the organizational centers for the microtubules (Lechler and Fuchs, 2007).

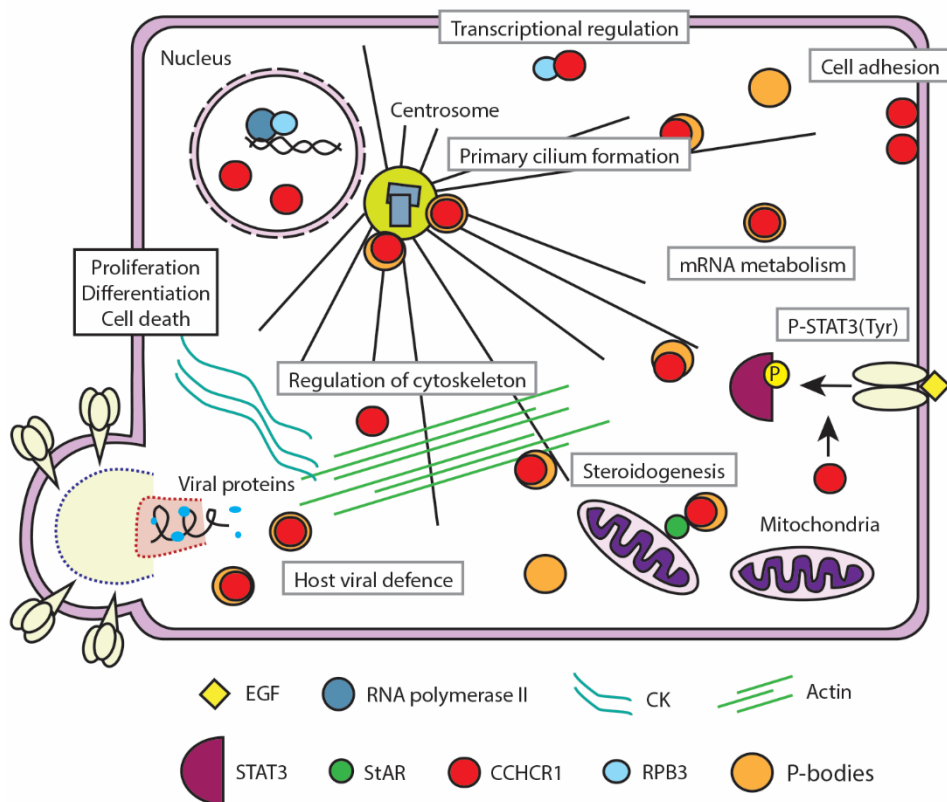


Figure 10 Possible functions of CCHCR1 in the cell. The figure is based on the literature, current interacting profile of CCHCR1, and the results in this thesis. CCHCR1 is suggested to play a role in several processes that influence proliferation, differentiation, and cell death. It is located in the centrosome, the main center for cell cycle control, and isoform-specifically in the P-bodies; centrosomal P-bodies control primary cilium formation, which suggests participation of CCHCR1 in this process. CCHCR1 has haplotype-specific effects on actin cytoskeleton, observed especially when the microtubulai are disrupted. P-bodies are linked to microtubules and actin cytoskeleton as well. In addition, P-body protein XRN1, which interacts with CCHCR1, regulates microtubule assembly. CCHCR1 interacts with mitochondrial StAR and is involved in steroidogenesis. Moreover, mitochondria, P-bodies, and microtubules interact as well. RPB3 (RNA polymerase II subunit 3) interacts with CCHCR1, which acts as its cytoplasmic docking site, therefore controlling transcription and differentiation. The RPB3-CCHCR1 interaction may also affect the mRNA decay pathway, for it has been shown that RNA polymerase II subunits (RPB4 and RPB7) can translocate from nucleus to the cytoplasmic P-bodies. CCHCR1 interacts with several viral proteins and P-body components are required for viral cycle completion. P-body components might also be involved in the host viral defence via interacting with several viral RNAs or proteins. We also demonstrated the isoform-specific effect of CCHCR1 on STAT3 Tyr705 phosphorylation, which in turn regulates many of the processes already mentioned above. Remade and modified from Ling et al. 2014.

Centrosome regulates the organization of microtubules, therefore modulating the shape and size of the cell (Badano et al., 2005). Stable overexpression of CCHCR1 resulted in isoform- and haplotype-specific morphological changes in cell size and shape. This could be caused by the alterations in the cytoskeleton, which is also supported by previous microarray data from transgenic CCHCR1 mice (Elomaa et al., 2004). Here we demonstrated that overexpression of CCHCR1 affects the arrangement and expression of actin, vimentin, and cytokeratins. Nocodazole is an agent that inhibits the polymerisation of microtubule filaments and the treatment with it affected CCHCR1 expression and localization in the stable cell lines, suggesting that microtubules partially regulate CCHCR1 localization. Disruption of the microtubule cytoskeleton affects the actin cytoskeleton for they are dependent on each other (Enomoto, 1996). Interestingly, in the isoform 3 -overexpressing cell line, with Risk allele, the effect on actin cytoskeleton was seen after disruption of the microtubule cytoskeleton, causing formation of actin-rich clusters. The clusters resembled podosomes or invadopodia, which are actin-containing structures involved in cell migration and invasion. Vimentin is also present in the elongated mature invadopodia (Schoumacher et al., 2010). Similar clustering of vimentin after the disruption of the microtubules cytoskeleton, however, was not observable in our study. The expression of vimentin was downregulated especially in the isoform 3 -overexpressing cells. Not surprisingly, vimentin is involved in the cell proliferation and maintenance of cell shape and its gene expression is downregulated in the psoriatic skin (Henno et al., 2009; Lund et al., 2010; Mendez et al., 2010; Paccione et al., 2008).

The shared DEGs from all four overexpressing cell lines, detected by RNA-seq, highlighted regulation of actin cytoskeleton and focal adhesion, which were downregulated in both Risk cell lines. Several relevant genes, whose expression products may disturb cytoskeletal organization, were downregulated especially in Iso3Risk cells. These included talin-1 (*TLN1*) and fibronectin 1 (*FN1*), which were downregulated in Iso1Risk and both isoform 3 -overexpressing cells, whereas they were upregulated in the Iso1Non-risk cells. TLN1 plays a significant role in the assembly of actin filaments and in spreading and migration of cells and indirectly interacts with FN1 (Jiang et al., 2003), which mainly functions in cell adhesion (Muro et al., 2003) and was downregulated also in the lesional SG samples. Interestingly, CCHCR1 has been also suggested to affect adhesion in

psoriatic skin where it was expressed in keratinocytes that had absent membranous β -catenin staining (Suomela et al., 2003)

The expression levels and patterns of various cytokeratins are also changed in the psoriatic skin (Henno et al., 2009; Leigh et al., 1995). Previous microarray results from transgenic mice suggest that CCHCR1 might regulate the expression of cytokeratins (Elomaa et al., 2004). The expression of cytokeratins was decreased most prominently in the Iso3Risk-overexpressing cells, which is consistent with previous results from experiments with transgenic mice that overexpress CCHCR1-Iso3Risk and exhibited the keratins as the most strongly downregulated gene group when compared with Iso3Non-risk mice (Elomaa et al., 2004). Here we also showed that the overexpression of CCHCR1 affects the expression of KRT17, a hallmark and plausible auto-antigen for psoriasis (de Jong et al., 1991; Gudmundsdottir et al., 1999; Leigh et al., 1995; Shen et al., 2006). The expression of KRT17 was upregulated in the Iso1Non-risk-overexpressing cells and the expression was stimulated by EGF in both cell lines overexpressing isoform 1 but not in the cell lines overexpressing isoform 3. The silencing of CCHCR1 in HEK293 cells downregulated the *KRT17* expression. KRT17 is overexpressed in the psoriatic lesions and its expression is altered in experiments with transgenic mice overexpressing CCHCR1 (de Jong et al., 1991; Elomaa et al., 2004; Leigh et al., 1995; Shi et al., 2011). In psoriatic skin, it is suggested to promote epithelial proliferation, modulate immune responses, and to have antiapoptotic effects (Chang et al., 2011; Depianto et al., 2010). KRT17 is also suggested to regulate cell growth and size by promoting protein synthesis (Kim et al., 2006) and therefore the upregulation of KRT17 in Iso1Non-risk cells may also cause the increase in cell size.

CCHCR1 has been implicated to play a role in keratinocyte proliferation (Tiala et al., 2008), which led us to investigate the role of the different isoforms and haplotypes on proliferation. Isoform 3 -overexpression led to faster multiplication and with the Risk haplotype also showed slight increase in apoptosis. After synchronization the rate of apoptosis increased as the cell cycle progressed, suggesting failure to proceed from cell cycle check points, which may result from the cytoskeletal aberrations. Measuring proliferation in cells where the cells nuclei are varying in size was challenging; methods relying on the amplification of DNA could not detect proliferation as the Iso1Non-risk cells seem to form multinucleated cells but fail to go through cytokinesis, whereas the size of the

nucleus is smaller in the isoform 3 -overexpressing cells that divide more vigorously, but as in the case of Iso3Risk cells, also fail to propagate further and go to apoptosis. Interestingly, the expression of anti-apoptotic peptide encoding humanin-like genes were among the DEGs detected by RNA-seq.

The localization of CCHCR1 is thus dynamic and it traffics between the centrosome and cytoplasm. CCHCR1 was recently identified as a component of cytoplasmic P-bodies that regulate various posttranscriptional processes (Ling et al., 2014). P-bodies are cytoplasmic sites for regulation of mRNA turnover in a post-transcriptional manner and their major functions involve mRNA degradation and surveillance, translational repression, and RNA-mediated gene silencing (Eulalio et al., 2007). Interestingly, cytoplasmic P-bodies are able to traffic to and from the centrosome, along microtubules, and some stationary P-bodies even reside at the centrosome (Aizer et al., 2008; Moser et al., 2011). We verified the location for both CCHCR1 isoforms but observed isoform-specific effects on the P-body localization (unpublished data). The isoform 1 -overexpressing cells, especially Non-risk, illustrated only occasional colocalization of CCHCR1 with the P-body markers, whereas the isoform 3 -overexpressing cells exhibited clear colocalization. The interaction with P-body marker EDC requires the N-terminus of CCHCR1 isoform 3 (Ling et al., 2014). Isoform 1 is 89 amino acids longer than isoform 3, at its N-terminus. Therefore, the additional amino acids might affect the localization of the P-bodies at the centrosome. Interestingly, GO analysis from the RNA-seq data illustrated enrichment of DEGs specific to the isoform 3 -overexpressing cells on transcription regulation. RNA degradation and mRNA surveillance pathway were upregulated especially in the Iso3Non-risk cells. Interestingly, negative regulation of transcription from RNA polymerase II promoter was among the upregulated functions that were shared in all the cell lines overexpressing CCHCR1, which is supported by the interaction of CCHCR1 with its subunit RPB3 (Corbi et al., 2005). Moreover, RPB3 regulates the expression and compartmentalization of vimentin (Corbi et al., 2010), which implicates that the effect of CCHCR1 on vimentin organization could also be mediated through its interaction with RPB3.

Our results from the stable cell lines strengthen previous findings that implicate regulation of the EGFR-STAT3 signaling pathway by CCHCR1. It has been also previously shown that EGF stimulates CCHCR1 expression in HaCaT cells and in

skin cancer CCHCR1 and EGF receptor (EGFR) are expressed in same areas (Tiala et al., 2007). In transgenic mice, the expression of constitutively active Stat3 leads to a psoriasis-like skin phenotype and the keratinocytes within psoriatic skin are characterized by activated STAT3 (Sano et al., 2005; Sano et al., 2008). Here we showed that EGF treatment induces the expression of CCHCR1 in the stably overexpressing CCHCR1 cell lines, both on mRNA and protein level. Interestingly, EGF is known to stimulate the expression of several genes, such as β -catenin and thrombospondin-1, through post-transcriptional mechanisms (Lee et al., 2010; Okamoto et al., 2002). We suggest that the increase in CCHCR1 expression is based both on mRNA and protein stabilization. We also showed that CCHCR1 regulates EGF-induced STAT3 phosphorylation in an isoform-specific manner: STAT3 tyrosine 705 phosphorylation was disturbed in the isoform 3 - overexpressing cells, whereas the CCHCR1 isoform 1 slightly activated the STAT3 phosphorylation, even without EGF-induction. The RNA-seq experiment, however, exhibited upregulation of ErbB signaling mainly in the Iso3Non-risk cells and downregulation in Risk cells. ErbB signaling is linked to psoriasis in several studies (Ainali et al., 2012; Piepkorn et al., 2003) and was enriched also in our lesional SG samples. *AREG* encodes a protein that belongs to the EGF family and binds EGFR as well. It associates with a psoriasis-like skin phenotype (Schneider et al., 2008), shows increased protein expression in lesional skin (Piepkorn, 1996), and might be affecting the integrity of cell-cell junctions in psoriasis (Chung et al., 2005). In our overexpressing cell lines, *AREG* was upregulated in the Iso1Non-risk cells and might also drive the STAT3 phosphorylation.

The silencing of CCHCR1 in HEK293 cells decreased the tyrosine 705 phosphorylation. Tyrosine 705 phosphorylation of STAT3 is suggested to be crucial for its nuclear translocation (Amin et al., 2004; Reich and Liu, 2006). Constitutive activation of STAT3 in psoriatic skin may result from the persistent stimulation of EGFR for its multiple ligands, including HB-EGF, are increased in psoriatic skin (Sano et al., 2008; Schneider et al., 2008; Yoshida et al., 2008b). STAT3 regulates various processes, such as cell proliferation, differentiation, apoptosis, and differentiation of the TH17 helper T cells, in the skin (Sano et al., 2008; Yang et al., 2007). Interestingly, KRT17 expression is also upregulated by STAT3 (Shi et al., 2011), suggesting that the induction of KRT17 in HEK293 cells overexpressing CCHCR1 isoform 1 with Non-risk allele, is regulated through this pathway. Furthermore, STAT3 also controls post-transcriptional processes and

modulates centrosome doubling by regulating γ -tubulin levels (Metge et al., 2004). Furthermore, it also regulates cell migration directly by binding stathmin, a depolymerization agent of microtubules (Ng et al., 2006).

We compared the expression profiles of the psoriatic SGs and the CCHCR1-overexpressing cell lines to illustrate further the link between CCHCR1 and psoriasis. The two different study groups shared several functions and pathways, including the above-mentioned EGF receptor signaling pathway. In addition, the shared DEGs were enriched in functions and pathways related to e.g. metabolism of proteins, Wnt and MAPK signaling, apoptosis, and cell cycle, most of which are well described in psoriasis. Pathways associated with innate immunity were highlighted as well, including the RLR and NLR signaling pathway and pathways related to infections. *IL8*, an important mediator of the immune reaction in the innate immune system response, was induced in the isoform 1 -overexpressing cells. Furthermore, *IL8* and *STAT3* regulate each other in certain conditions, including psoriasis (de la Iglesia et al., 2008; Kanda et al., 2011; Zhang et al., 2012). The pathogenic link between the immune system and CCHCR1, in cultured keratinocytes, was also suggested in the earlier studies, where *IFN- γ* was shown to downregulate *CCHCR1* expression. Interestingly, P-bodies are as well associated with innate immunity via colocalization with apolipoprotein B mRNA editing enzyme *APOBEC3G* (Wichroski et al., 2006), which was downregulated especially in the cells with overexpression of CCHCR1 isoform 3, with the Risk haplotype. *APOBEC* family of proteins has been suggested to play a role in innate anti-viral immunity (Takaori, 2005) and the gene *APOBEC3A* was highly upregulated in our psoriasis lesional samples.

CONCLUSIONS AND FUTURE PROSPECTS

RNA-seq, with RNA spike-in normalization, revealed more accurate expression profiling in different sample types with varying amounts of RNA. Combined with the use of skin graft samples it allowed improved recognition of altered transcripts, functions, and pathways in psoriasis. Compared with the previous transcriptomics studies on psoriasis, our approach provided more information about the transcriptional dysregulation in the epidermis. A deeper understanding on the components of NOD-like receptor signaling pathway and inflammasome activation in keratinocytes is a good example of the sensitivity of the method. Some of the components have been associated with psoriasis in previous studies, yet the exact composition and activation mechanisms of inflammasomes in psoriasis have remained unclear. Our RNA-seq and immunostainings strengthen previous findings on psoriasis, suggesting that all components needed for the active inflammasome are present in keratinocytes of the epidermis and are activated in the lesional skin. The inflammasome type and factors leading to its activation in psoriasis, however, remain to be determined. The 5' end RNA-seq method allows the precise determination of transcription start sites as well. Therefore, whether aberrant gene expression patterns that promote the pathogenesis of psoriasis arise from alternative promoter usage, remains to be investigated.

Here we presented that CCHCR1 localizes at the centrosome and verified its localization in the P-bodies. Our experiments show haplotype-specific effects of CCHCR1 on cytoskeletal organization and cell proliferation, functions relevant to the pathogenesis of psoriasis (Figure 11). Furthermore, our results suggest that CCHCR1 might function in EGFR-STAT3 signaling and innate immunity, which are functions previously implicated in psoriasis as well. In addition, the RNA-seq revealed isoform and haplotype-specific effects on expression profiles of different CCHCR1 cell lines, which strengthens the role of CCHCR1 as an effector gene in the pathogenesis of psoriasis. Furthermore, CCHCR1 plays a role in several pathways that have been associated with psoriasis. Interestingly, the most dramatic changes in gene expression were observable in the isoform 3 - overexpressing cells but also the Non-risk and Risk alleles had opposing effects. The observation that CCHCR1 influences multiple cell signaling pathways may result from its possible role as a centrosomal P-body protein, which suggests a role in post-transcriptional regulation as well as a role in regulation of the cell

cycle. Its exact function in these cellular compartments and effect in psoriatic lesions remains to be studied further. The identification of dysregulated pathways in the psoriatic epidermis and the cellular functions of the psoriasis candidate gene *CCHCR1* could provide information for novel medications, plausibly targeting the innate immunity pathways.

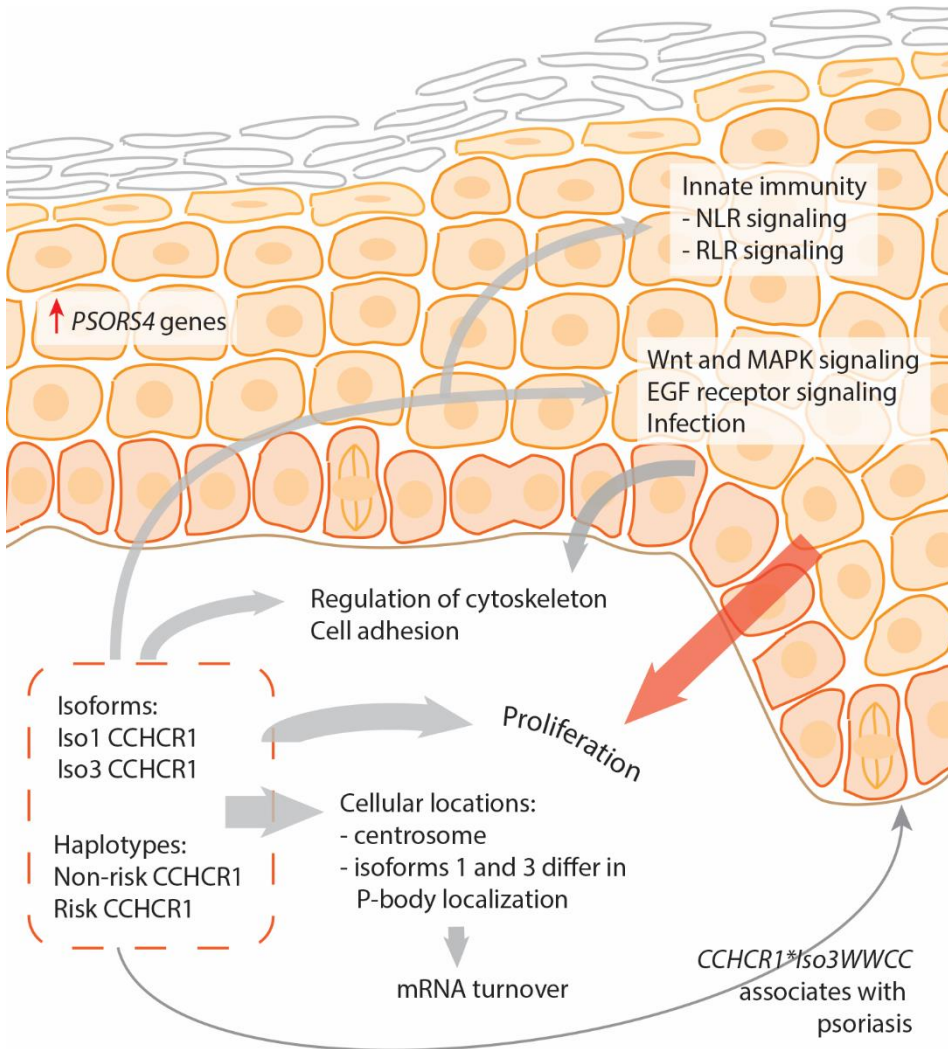


Figure 11 Summary of the main conclusions in this thesis. In this thesis, we genotyped a novel SNP that enables the translation of a longer isoform (Iso1) and redefined the psoriasis-associated haplotype of *CCHCR1* as **Iso3WWCC*. This suggests that, in some of the psoriasis patients, the absence of isoform 1 might have an effect on the onset or the propagation of the disease, in addition to the previously described Risk (**WWCC*)

haplotype. In functional studies of CCHCR1, indeed, had isoform-specific effects on e.g. proliferation, which is the main characteristics of the disease. In healthy skin, CCHCR1 is expressed in the keratinocytes at the basal layer of the epidermis. In the thickened psoriatic epidermis, its expression is altered: it is expressed also in supra-basal layers above the tip of the dermal papillae. We found that its localization is dynamic in the cell and it locates especially at the centrosome, and additionally at the P-bodies, while possibly having an isoform-specific effect on their localization in the cell. Both centrosomes and P-bodies are physically connected to the cytoskeleton that functions as a scaffold for the regulation of e.g. mRNA turnover, cell division, cell adhesion, and transport of organelles, such as mitochondria. Our previous results and the present RNA-seq suggest that CCHCR1 regulates expression of cytoskeletal components and functions related to cytoskeleton, such as cell adhesion. These functions, such as regulation of actin cytoskeleton, were altered also in our RNA-seq of the psoriatic skin. Furthermore, CCHCR1 may control the mRNA turnover at P-bodies by regulating RNA surveillance and degradation. This may affect a wide variety of diverse biological processes. RNA-seq identified several pathways for CCHCR1 which are relevant for the pathogenesis of psoriasis, such as epidermis development, Wnt, ErbB, and MAPK signaling, and innate immunity. The RNA-seq of psoriatic lesional epidermis highlighted the importance of the activation of the innate immunity pathways in the keratinocytes. In addition, our RNA-seq of the non-lesional skin of the psoriasis patients redefined the upregulation of *PSORS4*-derived transcripts.

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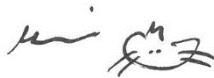
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APPENDIX: Original publications