SAPIENZA
UNIVERSITÀ DI ROMA

The role of Notch signaling and epidermal cytokines in the Hailey-Hailey disease pathogenesis and in the DNA damage response

PhD Programme: INNOVATION IN IMMUNO-MEDIATED AND HEMATOLOGICAL DISORDERS

Curriculum: IMMUNOLOGY

Department: Molecular medicine

XXXIII CYCLE

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A.A 2019-2020

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Abstract

Hailey-Hailey disease (HHD) is a skin disorder linked to mutations in the ATP2C1 gene encoding a Ca^{2+}/Mn^{2+} ATPase. ATP2C1 loss of function in keratinocytes leads to the loss of cell-to-cell adhesion in the suprabasal skin layers. The disorder is manifested as blistering skin lesions that do not heal and susceptible to microbial infections. Additionally, patients with squamous/basal cell carcinomas arising in the skin lesions have been described. There is no long-term treatment known to be effective in reducing the manifestations of HHD. The identification of compounds with fewer side effects compared to those used in the SOC treatment is highly desirable and could be reached by understanding the molecular mechanisms underlying the disorder. We previously found that HHD keratinocytes have increased oxidative stress that is associated with impaired proliferation, differentiation and DNA damage repair; these aspects are linked to the decreased action of some detoxifying systems and/or to deregulation in molecular pathways crucial for skin homeostasis such as the Notch1 signaling. Notch1 signaling influences many physiological aspects of the skin including differentiation, wound repair and also the DNA damage response. Moreover, HHD keratinocytes are characterized by deregulation in cytokines expression.

The aims of my PhD thesis were designed to dissect the relationship between DNA damage engagement, inflammatory signaling, oxidative stress and Notch1 deregulation in HHD development.

I. INTRODUCTION

1. Barrier function of the skin: “la raison d’etre” of the epidermis

The skin is the largest organ of the human body, accounting for approximately 16% of total body weight. The skin's structure is made up of an intricate network which serves as the body’s initial barrier against pathogens, UV light, chemicals, and mechanical injury (Wickett R.R. and Visscher M.O., 2006). The skin also has important immune and sensory functions, helps to regulate body temperature, and synthesizes vitamin D. Any discussion of the structure of skin will necessarily refer to layers. The various layers of the skin work in concert to provide strength and flexibility and perform the multiple functions of the skin. It is made up of three layers, the epidermis, dermis, and the hypodermis, all three of which vary significantly in their anatomy and function (Yousef H. and Sharma S., 2017). The epidermis is the outermost layer of the integument. The barrier function of the skin has been called “la raison d’etre” of the epidermis. The epidermal barrier serves to limit passive water loss from the body, reduce the absorption of chemicals from the environment and prevent microbial infection (Madison K.C., 2003).

1.1 Epidermis structure

The epidermis is a stratified squamous keratinized epithelium; it is a self-renewing tissue: a single, adult skin stem cell has sufficient proliferative capacity to produce enough new epidermis to cover the body surface (Rochat A. et al, 1994). Keratinocytes are the most frequent cells constituting the epidermis (Brody I., 1960). Other less-abundant and nonepithelial cells are interspersed among the keratinocytes in specific locations. These cells are the melanin-producing melanocytes, tactile Merkel cells (Merkel F.S., 1875) and antigen-presenting Langerhans cells (Langerhans P.,1868).

The epidermis is divided into several layers, or strata, starting with the *stratum basale* (basal layer) just above the dermis proceeding upward through the

stratum spinosum (prickle layer) and the *stratum granulosum* (granular layer) to the top layer, the *stratum corneum* (Wickett R.R. and Visscher M.O., 2006) (**Fig.1**)

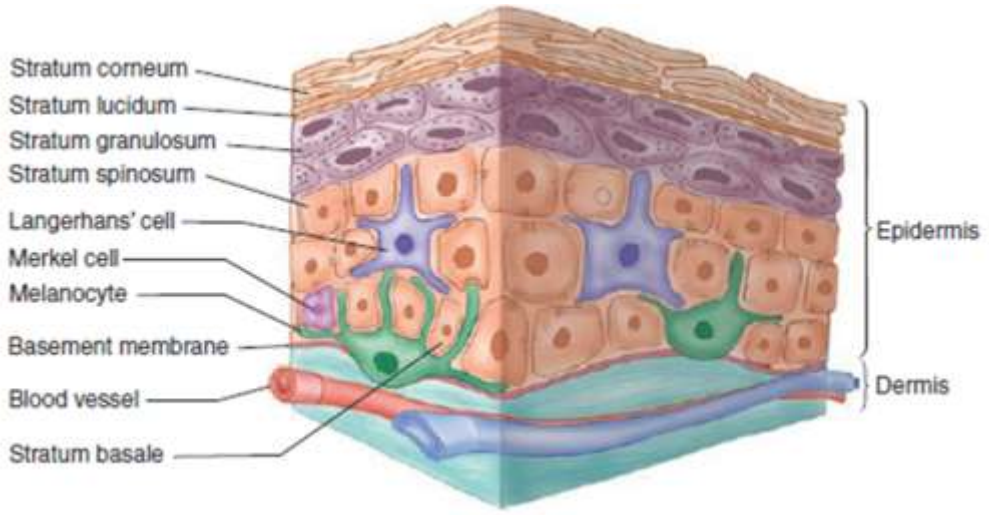

Figure 1 Epidermis layers (Gartner L.P. and Hiatt J.L., 2014)

The *stratum basale* consists of a single layer of basophilic low-columnar to cuboidal-shaped keratinocytes with large nuclei. They are firmly attached to the basement membrane with hemidesmosomes and to lateral and upper adjacent cells with desmosomes. Numerous mitotic figures indicate that the layer is germinal and is the source of the keratinocytes of the upper layers (Mistry D.S., Chen Y. and Sen G.L., 2012).

Some of these proliferated cells stay attached to the basal lamina as stem cells while others, known as transit-amplifying, differentiate to spinal keratinocytes of the *stratum spinosum* and push the overlying cells towards the surface (Barrandon Y. et al, 1987).

The *stratum spinosum* is the thickest layer of the epidermis. It consists of several layers of cells. The cells in the basal layers of the *stratum spinosum* show mitotic activity similar to the ones in *stratum basale*; together, they are often referred to as the Malpighian layer. The majority of the cells of the *stratum spinosum* are polyhedral-shaped keratinocytes. They flatten as they approach the surface.

They produce 10-nm tonofilaments made out of keratins. Synthesis of tonofilaments increases, and they group together into bundles forming tonofibrils as the cell moves towards the surface (Brody I., 1960). Tonofibrils terminate in desmosomes, which are the units of strong intercellular junctions in between the spinous cytoplasmic protrusions of the keratinocytes. Adjacent keratinocytes interdigitate with each other and form numerous intercellular bridges among them with these spinous extensions and firmly join with each other by desmosomes. The term “spinous” refers to this pattern of alignment. Keratin is essential for the protection of skin (Allen T.D. and Potten C.S., 1975). Langerhans cells (antigen-presenting dendritic cells) derive from monocytes of bone marrow and represent the mononuclear phagocyte system in skin. They scatter mostly in the *stratum spinosum* among the keratinocytes of the Malpighian layer. These dendritic cells are capable of binding, processing, and presenting antigens to Tcells, and they initiate the response against foreign antigens in a similar way as immune dendritic cells found elsewhere (Girolomoni G. et al., 1990; Stingl G. et al., 1977).

The *stratum granulosum* consists of 1-5 layers of flattened polygonal granulated cells. These cells undergo final cell differentiation of the uppermost keratinocytes of the *stratum spinosum*. They still have nuclei. The large, irregularly shaped and uncoated keratohyaline granules made up of filaggrin and other proteins related to the keratins of tonofibrils fill the cytoplasm. They do not have a limiting membrane. The other characteristic feature of the cytoplasm is membrane-bound small lamellar lipid granules. The cells discharge their granular content to the intercellular spaces by exocytosis. The lipid-rich substance spreads to fill the intercellular spaces and forms the major epidermal permeability barrier of the skin (Elias P.M., 2012).

The *stratum corneum* is composed of 15-20 layers of flattened highly keratinized cells filled with keratins. The defensive functions of the skin reside primarily in the *stratum corneum*. The cells contain only fibrillar and amorphous proteins embedded in an amorphous matrix. The dead cells continuously exfoliate from the surface of the epidermis (Elias P.M., 2012).

The cellular architecture of the epidermis is essential for its barrier and protective functions, and this architecture is perturbed in several human genetic skin disorders, including degenerative blistering diseases, such as epidermolysis bullosa simplex (EBS) and palmoplantar keratoderma, as well as in epithelial skin tumours and cancers. In the metabolically active layers of the epidermis, intercellular adhesion is accomplished by two types of intercellular junction: the desmosome and the adherents junction. Both of these intercellular junctions are essential for epithelial sheet formation and to preserve skin integrity (Green K.J. et al, 2000; Vasioukhin V. et al, 2000).

Desmosomes are also defective in patients with Darier's disease and Hailey-Hailey disease who have impaired calcium homeostasis because of mutations in the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) (Sakuntabhai A. et al. 1999; Dhitavat J. et al. 2003) and in the Golgi secretory pathway Ca2+/Mn2+ ATPase (hSPCA1) (Sudbrak R. et al., 2000; Hu Z. et al., 2000).

1.2 Calcium regulation of keratinocytes differentiation

Calcium is the major regulator of keratinocytes differentiation *in vivo* and *in vitro*. A calcium gradient within the epidermis promotes the sequential differentiation of keratinocytes as they traverse the different layers of the epidermis to form the permeability barrier of the *stratum corneum*. Calcium promotes differentiation by both outside–in and inside–out signaling. A number of signaling pathways involved with differentiation are regulated by calcium, including the formation of desmosomes, adherents junctions and tight junctions, which maintain cell–cell adhesion and play an important intracellular signaling role through their activation of various kinases and phospholipases that produce second messengers that regulate intracellular free calcium and PKC activity, critical for the differentiation process. The calcium receptor plays a central role by initiating the intracellular signaling events that drive differentiation in response to extracellular calcium (Bikle D.D. et al., 2012). Alterations of calcium gradient within the epidermis and, in turn, of the differentiating process, are involved in the pathophysiology of

several dermatological diseases including psoriasis, atopic dermatitis and also Hailey-Hailey's disease (Kellermayer R., 2005) **(Fig.2)**.

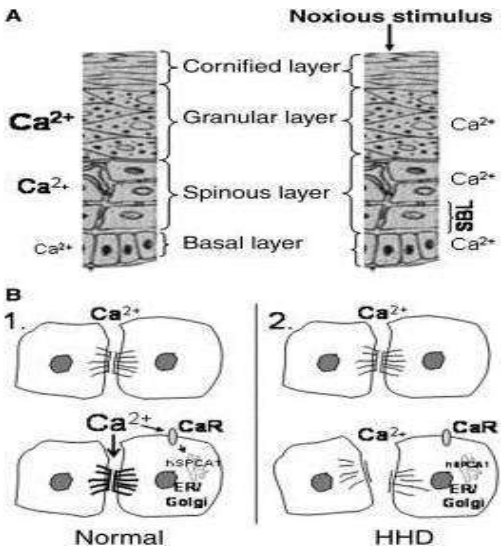

Figure 2 Calcium gradient within the epidermis in pathophysiological conditions (Kellermayer R., 2005).

As mentioned above, there are several genomic and nongenomic pathways through which calcium induces epidermal differentiation. An example of a rapid and nongenomic pathway is represented by the redistribution to the membrane of desmoplakin to form desmosomes, occludins and claudins to form tight junctions and E-cadherin with its associated catenins and kinases to form adherens junctions. These membrane complexes provide not only adhesion between cells but also a signaling complex that participates in changes in actin distribution and sustained increases in intracellular calcium (Hennings H. and Holbrook K., 1983; Niessen C.M., 2007).

With the sustained increase in intracellular calcium, genomic pathways are triggered. The cells begin to express in sequential fashion K1 and K10, involucrin and transglutaminase-I and loricrin and filaggrin (Elsholz F. et al., 2014). A number of these genes (e.g., involucrin and K1) have known response elements, such as activator protein 1 (AP-1) sites for calcium and phorbol esters, acting at least in part by PKC activation.

A crucial role in the calcium-mediated keratinocytes differentiation is played by a class GTPbinding protein-coupled receptors called CaR; CaR receptors are expressed in the keratinocytes of the supra-basal layers and are responsible for sensing extracellular calcium levels. The CaR receptors stimulation leads to the activation of phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which activate protein kinase C (PKC) and mobilize intracellular Ca^{2+}. In particular, IP_3 binds to IP_3R located on the intracellular Ca^{2+} stores, mainly the endoplasmic reticulum and the Golgi apparatus. As consequence, an acute and transient increase in intracellular calcium concentration is triggered (Berridge M.J., 1993).

The role of ER and Golgi in modulating cytosolic calcium concentration and epidermal differentiation is illustrated by two skin disorders, Darier's disease (DD) and Hailey-Hailey disease (HHD) (Foggia L. and Alain H., 2004). These diseases are caused by inactivating mutations in ATP2A2 and ATP2C1 genes that encode the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) and the SPCA1, respectively. Keratinocytes from the patients' skin manifest dysregulated cell adhesion and differentiation due to the loss of the ability of ER and Golgi to store Ca^{2+} (Foggia L. and Alain H., 2004); because of the important role played by calcium in the epidermal homeostasis regulation, mutations in SERCAs and SPCAs mainly affect the skin despite being ubiquitous expressed. Alternatively, even if not yet completely clear, in keratinocytes there may be no compensatory mechanisms that protect other types of tissue from the partial or total absence of these proteins (Missiaen L. et al., 2006).

1.3 Oxidative stress and DNA Damage response as keratinocytes differentiation regulators

Reactive oxygen species (ROS) have historically been viewed as toxic metabolic by-products and causal agents in a myriad of human pathologies. Whereas mitochondrial ROS production has commonly been thought of solely as the result of inefficiencies in the electron transport chain, a role for mitochondrial ROS in

the propagation of cellular signaling pathways has emerged (Hamanaka R.B. and Chandel N.S., 2010). Mitochondrial ROS generation is required for the propagation of numerous cellular signaling pathways including those regulating tumorigenesis (Weinberg F.R et al., 2010) immune responses (Zhou R. et al., 2011) and cellular adaptation to stresses such as hypoxia (Chandel N.S. et al., 1988). Increasing evidence suggests that mitochondria and mROS generation play key roles in cellular differentiation programs. Cellular mitochondrial content and oxidative capacity are increased when mouse embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or mesenchymal stem cells are induced to differentiate (Facucho-Oliveira J.M., 2007). Furthermore, increased cellular ROS levels and oxidation products correlate with the differentiation of ESCs and iPSCs as well as mesenchymal, neural, and epithelial stem cells (Yanes O. et al., 2010) while inhibition of ROS production prevents differentiation (Ito K. et al., 2007). Thus, increased mitochondrial and oxidant content appears to correlate with stem cell differentiation while low mitochondrial mass and oxidant content correlates with stem cell maintenance.

As already mentioned, the epidermis is a self-renewing stratified squamous epithelium and is thus regulated by stem cell populations. To maintain epidermal homeostasis, cells within the proliferative basal layer withdraw from the cell cycle and differentiate as they process outward through the suprabasal layers (Fuchs E., 2009). Multiple transcriptional networks are associated with differentiation within the epidermis including Notch, p63, C/EBP, and AP2 (interfollicular epidermis) and β-catenin (HF) (Blanpain C. and Fuchs E., 2009). In order to test the hypothesis that mitochondria play an active role in the regulation of epidermal homeostasis, as well as in keratinocytes differentiation, Hamanaka and colleagues conditionally deleted the mitochondrial transcription factor A (TFAM) in undifferentiated keratinocytes. TFAM is required for the replication and transcription of the mitochondrial genome, and cells lacking TFAM are unable to conduct oxidative phosphorylation or produce mROS. Authors reported that mice conditionally lacking TFAM (TFAM cKO) in epidermis developed an epidermal barrier defect contributed to their perinatal mortality. TFAM cKO mice keratinocytes displayed signs of impaired keratinocytes differentiation both *in vivo*

and *in vitro*; moreover, differentiation of wild-type keratinocytes was inhibited by antioxidant treatment, and differentiation marker expression in TFAM cKO cells could be partly restored by treatment with exogenously applied H_2O_2, clearly demonstrating that oxidative signaling promotes keratinocyte differentiation and that mROS act as pro-differentiation signals and are key upstream regulators of stem cell fate decisions (Hamanaka R.B. and Chandel N.S., 2013).

1.3.1 The role of transcription factor Nrf2

Due to its accessibility, the skin is permanently exposed to harmful environmental influences and is highly susceptible to the effects of different stimulants. UV irradiation, xenobiotics, and thermal stress disturb cell metabolism and consequently lead to the increase in reactive oxygen species (ROS) generation and to redox imbalance. Despite low levels of ROS are required for efficient intracellular signalling, an excessive accumulation of ROS and/or a reduction in the antioxidant capacity contribute to cell aging, severe cell damage, and even neoplastic transformation (Gegotek A., 2015). Cells have developed several strategies to protect themselves against these insults. A major mechanism in the cellular defence against oxidative stress is activation of the Nrf2-antioxidant response element signaling pathway, which controls the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant capacity (Nguyen T. et al., 2009).

The transcription factor Nrf2 (nuclear factor erythroid derived 2, like 2) belongs to the “cap‘n’collar” (CNC) protein family, which contains the motif called leucine zipper (bZip, basic Leucine Zipper). This family has three-dimensional structures that allow the formation of dimers with other proteins containing bZip domain. The family of transcription factors containing bZIP domain is also characterized by a basic region, which binds via hydrogen bonds to the large groove of the DNA (Konstantinopoulos P.A. et al., 2011).

Under physiological conditions, Nrf2 encoding gene is under constant expression, as a result of which Nrf2 molecule is permanently biosynthesized. However, its level in the cytoplasm is regulated by the formation of Nrf2-Keap1-Cul3 complex (Shibata T.T. et al., 2007). Keap1 binds Nrf2 and therefore directly inhibits its activity, resulting in simultaneous Nrf2 ubiquitination catalyzed by Cul3. Binding of at least four molecules of ubiquitin to Nrf2 causes degradation of this molecule by the proteasome 26S. However, the oxidative condition in the cell leads to the oxidation of cysteine residues in Keap1 molecule, changing the conformation of the protein and causing dissociation of Nrf2 from complex. Free Nrf2 cannot be ubiquitinated and degraded. In turn, it is translocated to the nucleus, where it forms a complex with a small Maf protein and then is bound to the DNA in a characteristic sequence 5′-TGACnnnGCA-3′ labeled as antioxidant responsive element (ARE) and in consequence initiates the transcription of antioxidant genes (Gegotek A., 2015). Nrf2 cytoprotective action concerns mainly antioxidant enzymes such as glutathione S-transferase (GST), quinone reductase NAD(P)H (NQO1), UDP-glucuronosyltransferases (UGT), epoxide hydrolase (EPHX), γ-glutamylcysteine ligase (GCL), heme oxygenase-1 (HO-1), glutathione reductase (GR), thioredoxin reductase (TrxR), catalase (CAT), and superoxide dismutase (SOD) (McMahon M. et al., 2001).

Thanks to its antioxidant properties, NRF2 is a good target for antioxidant drugs. Some skin diseases, such as atopic dermatitis and psoriasis have been successfully treated through NRF2 enhancers (Schafer M. and Werner S., 2015).

1.3.2 DNA Damage Response

During their lifespan, cells are inevitably challenged by extrinsic and intrinsic stresses that threaten the integrity of their genomes. To survive these adverse conditions and pass on intact genetic information to subsequent generations, cells have evolved a highly organized and coordinated effort to ameliorate genotoxic stress called the DNA damage response (DDR). This response underlies the organismal ability to sense and signal problems in its DNA, to arrest

cell-cycle progression (cell-cycle checkpoints) and activate appropriate DNA repair mechanisms, or to eliminate cells with unrepairable genomes. The importance of the DDR network for the development and well-being of humans is illustrated by the large variety of diseases and cancer-predisposition syndromes that have been linked to mutations of DDR genes (Ciccia A. and Elledge S., 2010).

In contrast to the signal transduction pathways that are activated by ligands of receptor kinases, the DDR signaling pathway is activated by aberrant DNA structures induced by DNA damage or DNA replication stress. The sensors of this pathway are the proteins that directly recognize these aberrant DNA structures and activate the most upstream DDR kinases. The DDR signaling pathway consists of a protein kinase cascade as well as mediator proteins that facilitate the phosphorylation events within the DDR network. The effectors of the DDR signaling pathway are substrates of the DDR kinases that participate in a wide spectrum of cellular processes important for genomic stability, such as DNA replication, DNA repair, and cell-cycle control (Marechal A. and Zou L., 2013). In mammalian cells, the ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related), and DNA-PKcs (DNA-dependent protein kinase) kinases are the most upstream DDR kinases. In response to DNA damage, hundreds of proteins are phosphorylated at Ser/Thr-Glu motifs and additional sites in an ATM- or ATR-dependent manner, whereas DNA-PKcs appears to regulate a smaller number of targets and play a role primarily in nonhomologous end joining (NHEJ) (Matsuoka S. et al, 2007; Smolka M.B. et al., 2007; Bensimon A. et al., 2010). ATM and ATR also activate a second wave of phosphorylation through their activation of Chk1, Chk2, and MK2 protein kinases (Matsuoka S. et al., 1998; Reinhardt H.C. et al. 2007). ATM and ATR are the master transducers of DNA signals, and they orchestrate a large network of cellular processes to maintain genomic integrity. *In vivo* and *in vitro* studies also suggest that the DNA-damage specificities and functions of ATM and ATR are distinct. Whereas ATM is primarily activated by double-stranded DNA breaks (DSBs), ATR responds to a broad spectrum of DNA damage, including DSBs and a variety of DNA lesions that interfere with replication.

Although the DDR is fundamental to preserve genome integrity in response to several damaging assaults, recent studies in normal precursor or stem cells suggest that even the cellular differentiation programs are under the influence of DDR. The role of DDR in terminal differentiation programs is controversial and it seems to be highly context dependent. As example, DDR activation blocks differentiation in myoblasts and melanocytes, whereas promotes it in B lymphocytes and neuronal cells (Sherman M.H. et al., 2011). However, in the epidermal context the terminal differentiation is triggered by the DDR repression. One of the major signal pathways involved in keratinocytes differentiation is the Notch signaling; genetic ablation or activation of the pathway reveals that Notch signaling promotes epidermal differentiation (Rangarajan A., 2001). Moreover, Notch receptor binds and inactivates ATM kinase and that this mechanism is evolutionarily conserved in Caenorhabditis elegans, Xenopus laevis and humans. Activation of human Notch1 leads to reduced ATM signaling; Notch1 binds directly to the regulatory FATC domain of ATM and inhibits ATM kinase activity (Vermezovic J. et al., 2015). By using a calcium-induced keratinocytes differentiation model, Cialfi S. and colleagues demonstrate that the inactivation of both ATM and DDR is Notch1 dependent and that the DDR repression is involved in driving keratinocytes toward differentiation (Cialfi S. et al., 2016).

1.4 The Notch signaling

A central role in the maintenance of skin homeostasis is played by the genes of Notch family. The Notch signalling is an evolutionarily conserved pathway from *D. melanogaster* to vertebrates (Artavanis-Tsakonas S. et al., 1999), involved in cell fate differentiation and in development of different multicellular organisms. Notch signalling regulates cell survival, proliferation, apoptosis and self-renewal events (Bray S., 2006). In mammals, *notch* genes encode four large single-pass type I transmembrane proteins receptors that display both redundant and unique functions: Notch1, Notch2, Notch3 and Notch4 (Kopan R. and Ilagan M., 2009). Notch proteins are constituted by an extracellular domain (NECD), involved in the

interaction with DSL ligands, and an intracellular domain (NICD) responsible of the signal transduction.

The extracellular domain contains 36 tandem epidermal growth factor (EGF)-like repeats. The interaction with ligands requires some of these repeats. Moreover, many EGF-like repeats bind calcium ions, necessary for the structure and affinity of Notch in ligand binding (Kopan R. and Ilagan M., 2009). The EGF-like repeats are followed by three cysteine-rich Notch/Lin12 (LNR) repeats and a heterodimerization domain (HD). The LNR repeats together with the HD form the negative regulatory region (NRR), essential in preventing Notch activation in the absence of a ligand.

The intracellular domain contains several RAM (RBPJ association module) domains, for protein-protein interaction, a seven ankyrin repeats domain (Ank/Cdc10) with two different nuclear localization sequences (NLS) on both sides, and a transactivation domain. Instead, the C-terminal domain is characterized by the PEST domain [proline (P), glutamic acid (E), serine(S) and threonine (T)-rich motif], that presents a degron, a degradation signal, to regulate Notch stability (Artavanis-Tsakonas S. et al., 1999; Kopan R. and Ilagan M., 2009) **(Fig.3).**

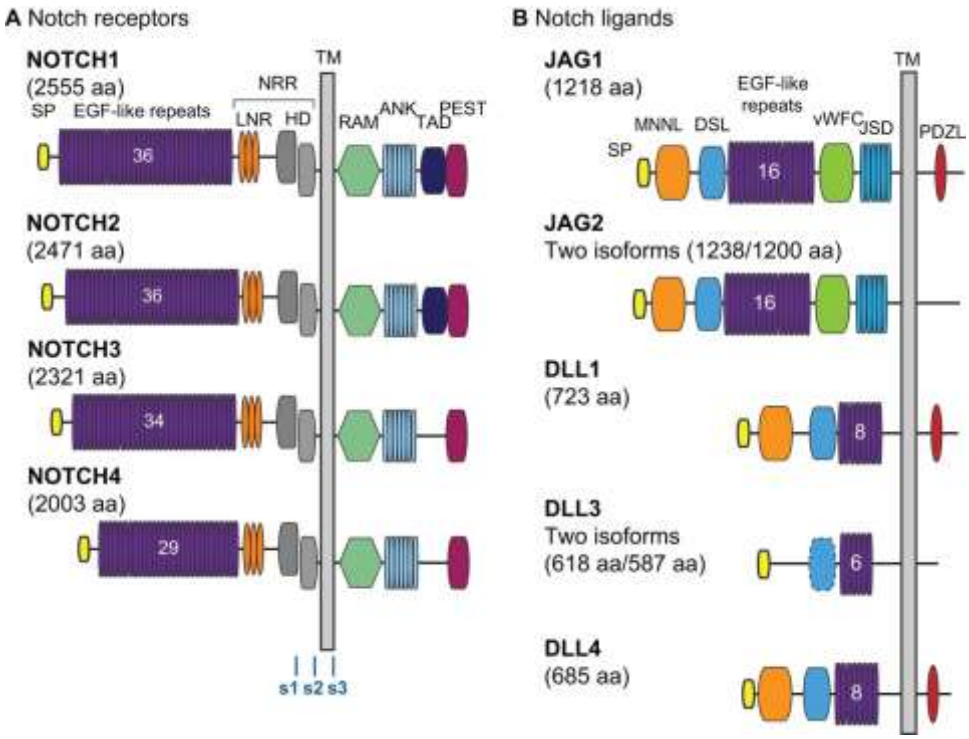

Figure 3 **Protein domain arrangement of human Notch receptors (A) and ligands (B).** ANK, ankyrin repeats; DLL, Delta-like protein; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor; HD, heterodimerization domain; JAG, jagged; JSD, Jagged Serrate domain; LNR, Lin-Notch repeats; MNNL, Notch ligand N-terminal domain; NRR, negative regulatory region; PDZL, PDZ ligand domain [PDZ, post synaptic density protein (PSD95)]; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; s, cleavage site; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain; vWFC, von Willebrand factor type C domain (Mašek J. and Andersson E., 2017)

The immature receptor undergoes different post-translational modifications, during maturation and trafficking to cell surfaces. Initially, Notch proteins are processed in the endoplasmic reticulum by O-fucosil transferase (O-Fut1) at the EGF-like repeats (Haines N. and Irvine K.D., 2003). O-Fut1 binds a residue of O-fucose to Notch, promoting its clustering to the surface (Allenspach E.J. et al., 2002). After this modification by O-Fut1, the immature precursor of Notch is cleaved by a furin-like convertase at a specific site (S1), during trafficking through the Golgi complex. Notch is converted as a heterodimeric receptor, with an extracellular domain (NECD), a trans-membrane domain (NTM) and an intracellular domain (NICD), held together by non-covalent interaction *via* a

heterodimerization domain (HD). The NECD undergoes O-linked glycosylation during Notch synthesis and secretion, which is crucial for proper folding of the Notch receptor and the interaction with its ligand DSL (Delta, Serrate, Lag-2) (Hori K. et al., 2013). The mature receptor is transported on the cell surface and held *in situ* by non-covalent interactions (Ntziachristos P. et al., 2014)(Fig).

The canonical Notch pathway involves trans-interactions between the receptors, expressed on the signal-receiving cells, and their specific DSL ligands, located on the signal-sending cells (Bray S., 2006; Hori K. et al., 2013). Different ligands cooperate with different notch receptors to determine the cell fate. Among them the DSL family (Delta/serrate/lag-2) are highly evolutionarily conserved.

The DSL ligands are type I transmembrane glycoproteins characterized by a DSL domain, at the N-terminal domain, involved in the binding with EGF-like repeats of Notch (Fiúza U. and Arias M.A., 2007). In addition, the ligands contain EGF-like repeats in a substantially variable number between the Delta and Serrate/Jagged1 family. Moreover, the serrate/jagged ligands are characterized by cysteine-rich domain (CRD) located between the transmembrane domain and EGF-like repeats (Ascano J.M. et al., 2003).

The Notch-ligand binding causes a conformational change in the receptor structure with the consequent exposure of two cleavage sites. In addition, endocytosis and membrane trafficking regulate ligand and receptor availability at the cell surface. Ligand endocytosis is also thought to generate mechanical force to promote a conformational change in the bound Notch receptor. This conformational change exposes site 2 (S2) in Notch for cleavage by ADAM metalloproteases. Indeed, the first cleavage is carried out by the family of metalloproteinase ADAM10/17, which recognize the site S2 placed in the extracellular region of Notch, determining its release (Aithal M. and Rajeswari N., 2013). The S2 cleavage mediated by ADAM metalloproteinases stimulates the presenilin complex (PS)/γ-secretase cleavage, in the S3 site placed within the intracellular domain. These events result in the release of an intracellular domain of Notch (NICD), which translocates to the nucleus (Bertrand F. et al., 2012) and forms a multiprotein complex with CSL proteins (CBF1 in mammals, RBPJ in the

mouse, “suppressor of hairless” in *D. melanogaster* and Lag1 in *C. Elegans*). At first, NICD binds CSL proteins with the RAM domain and, subsequently, with the Ank domain. When Notch signalling is turned off, CSL proteins inhibit transcription, together with co-repressor such as SMRT, NCoR and SHARP. The presence of Notch is able to derepress the promotor region by displacing corepressors and directly binding to CSL proteins (Doug B. and Kopan R., 2006). Notch requires Maml proteins and other transcriptional co-activators to drive the transcription of target genes, such as the Hes family (Bray S., 2006; Kopan R. and Ilagan M., 2009). Maml1 binds NICD and CSL only in a complexed dimer where the highly conserved N-terminal domain of Maml1 is fitting into a molecular groove formed by the Ank domain of Notch and specific residues of the CSL protein. In addition, Maml1 recruits additional cofactor, such as p300 and CDK8, to induce posttranslational modifications. Acetylation, phosphorylation and ubiquitination events, induced by these cofactors, mediate the binding affinity and stability of Notch transcriptional complex on target genes´ promoters) **(Fig.4)**.

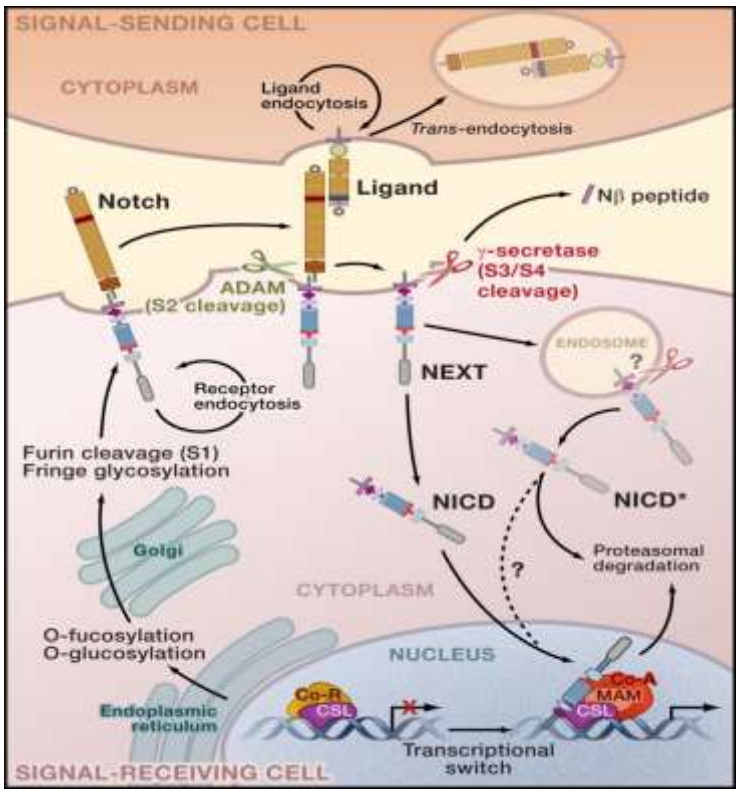

Figure 4 The Core of the Notch Signaling Pathway (Kopan R. and Ilagan M., 2009).

1.4.1 Epidermal Notch signaling

The epidermis is characterized by a polarized pattern of epithelial cell growth and differentiation. It is organized in several distinct overlying layers. Actively proliferating keratinocytes are limited to the innermost basal layer; within the proliferative compartment, at least two kinds of keratinocyte populations are present: multipotent “stem cells,” slow cycling but with an indefinite self-renewal potential and capable of generating all other types of growing and differentiating keratinocytes and “transient amplifying cells,” actively proliferating but capable of a limited number of cell divisions and already committed towards differentiation. The stem cell populations are not “fixed” in their properties, but are in a reversible transit towards surrounding transit amplifying cells that are in turn in equilibrium with cells that have withdrawn reversibly versus irreversibly from the cell cycle. The relative balance of these various populations could be controlled at any step of this dynamic process, and may be further determined by the surrounding cellular environment, i.e., the stroma and associated blood supply (Lefort K. and Dotto G.P., 2004). A pivotal role in controlling the balance between proliferative and differentiating signals is played by the Notch signaling; the Notch signaling activation results in the induction of a program of gene expression that can either suppress or promote differentiation in cell-type and context-dependent manner (Artavanis-Tsakonas S. et al., 1999). In normal epidermis, Notch1, Notch2 and Notch3 are all expressed suprabasally, indicating that their physiological role is mostly associated with keratinocyte differentiation. For example, blockade of Notch1 predisposes murine skin to basal cell carcinoma-like tumors. This observation could be explained by the fact that in normal keratinocytes, Notch activation induces cell cycle arrest (Rangarajan A. et al., 2001).

Consistently with the model that the Notch pathway plays an important role in regulating epidermal homeostasis, Notch1 deficiency in the epidermis results in a pleiotropic phenotype, with hair loss, hyperproliferation, deregulated expression of multiple differentiation markers and spontaneous basal cell carcinomas (Rangarajan A., et al., 2001*; Vauclair S., et al., 2005; Nicolas M. et al., 2003). Thus, consideration gathered from human and mouse epidermis indicates a simple model in which Notch signaling activation regulates epidermis

homeostasis by first promoting stem cell-transit amplifying cell transition and thereafter inducing a differentiation-associated growth arrest (Talora C. et al., 2008).

As already highlighted, levels of extra and intracellular calcium play a major role in keratinocytes growth/differentiation (Dotto G.P., 1999). Calcium dependent differentiation is further strengthened by the effects of calcium on Notch1 receptor activity (Lefort K. and Dotto G.P., 2004; Rangarajan A. et al., 2001*). High calcium levels induce Notch1 activation by enhancing its association with ligands and thus by stimulating the conformation changes needed for the receptor processing (Cordle J. et al., 2008). In addition, calcium influx stimulates the activity of proteins complex responsible for transmembrane receptors processing (Zoltowska K. and Berezovska O., 2018).

The molecular mechanisms by which Notch signaling regulates cutaneous development and homeostasis are complex and multifaceted and involve interactions with a variety of other signaling pathways (Nowell C. and Radke F., 2013). One mechanism by which canonical Notch signaling mediates its effects is both via the induction of CDKN1A/p21 and the repression of p63. Induction of p21 expression is one of the earliest cell cycle regulatory events underlying differentiation-associated growth arrest (Missero C. et al., 1995). In vitro studies on mouse epidermal keratinocytes indicate that the induction of CDKN1A/p21 by Notch is RBP-J dependent; the RBP-Jκ protein binds directly to the endogenous p21 promoter and both increased extracellular calcium and activated Notch1 induce p21 promoter activity through an RBP-Jκ-dependent mechanism (Rangarajan A. et al. 2001).

p63 belongs to the p53 family of transcription factors and exists as several isoforms that show distinct expression patterns (Yang A. et al. 2002). The ΔNp63α isoform is generally associated with epithelial stem/progenitor cells in stratified epithelial tissues (McKeon F., 2004) and plays an important functional role in the proliferation of progenitors during the process of stratification (Senoo M. et al. 2007). The Notch signaling represses p63 expression during differentiation, and overexpression of p63 in human epidermal keratinocytes

counteracts the prodifferentiation function of Notch (Nguyen B. et al. 2006). The mechanism by which Notch opposes p63 expression involves the regulation of interferon response factors (IRFs). In human and mouse epidermal keratinocytes, Notch-mediated repression of p63 is concomitant with the repression of IRF3/7, both of which prevent p63 down-regulation when overexpressed (Nguyen A. et al. 2006) **(Fig.5)**.

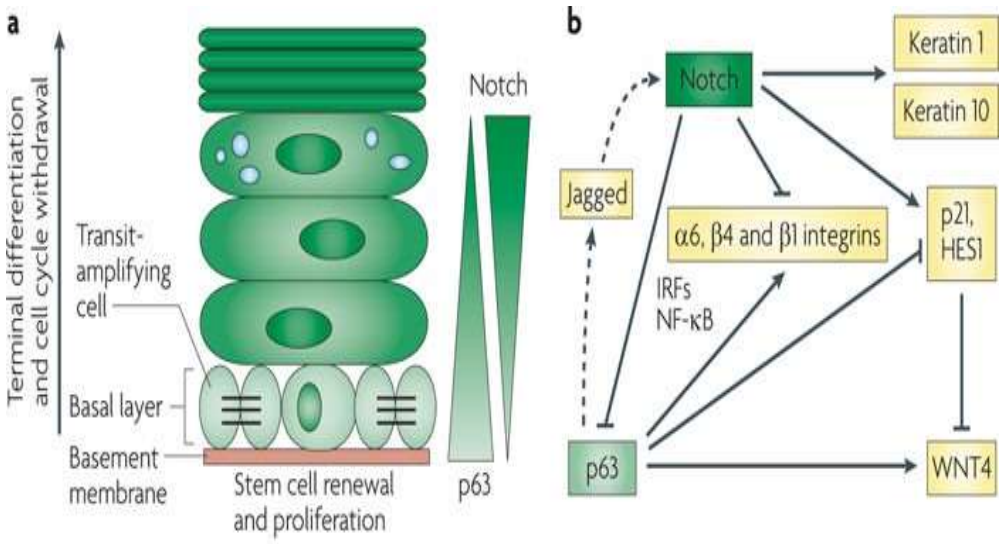

Figure 5 Regulation of the balance between self-renewing and committed cell populations mediated by the mutual antagonism between Notch e p63 (Dotto G.P., 2009).

2.The skin as an immune organ

One of the main functions of the skin is to protect the host from invasion, and it does so by employing physical barriers, antimicrobial peptides (AMPs), and an intricate network of resident immune and non-immune cells and skin structures (Nguyen A. and Soulika A., 2019) **(Fig.6)**.

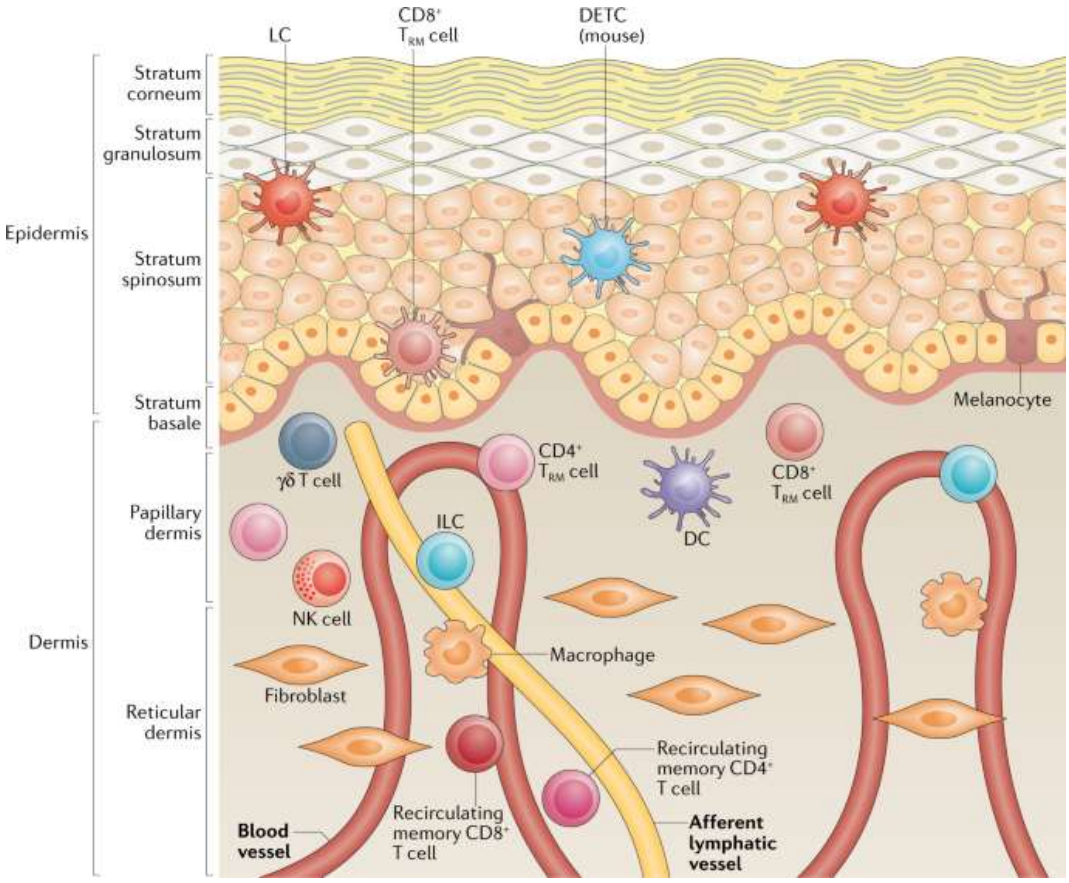

Figure 6 The most prevalent immune cell types that populate human epidermis are Langerhans cells (LCs) and $CD8^+$ tissue-resident memory T (T_{RM}) cells. The murine epidermis contains dendritic epidermal T cells (DETCs), which are absent in human epidermis. Both the mouse and human dermis are populated by dendritic cells (DCs), macrophages, innate lymphoid cells (ILCs), natural killer (NK) cells and $CD8^+$ T_{RM} cells. In murine dermis, γδ T cells play a prominent role in the production of IL-17, while in human skin αβ T cells are responsible. The functional coordination of these cellular mediators results in effective immune responses and a subsequent return to immune homeostasis (Ho A. and Kupper T., 2019).

2.1 Skin resident immune cells

Skin-resident immune cells promote tissue function in homeostasis and act as sentinels by actively sampling environmental antigens. Both myeloid and lymphoid cell subsets are found in the skin in steady state. Some of these resident immune cells migrate to lymph nodes to either induce peripheral tolerance to tissue self-antigens or initiate robust immune responses. In the event of a challenge, such as infections or tissue injury, immune cells resident in the skin and those infiltrating from the periphery interact to create an intricate defence network to resolve the insult and restore the tissue to its original state (Nguyen A. and Soulika A., 2019).

Skin-resident myeloid cells include: Langerhans cells, dermal dendritic cells, macrophages, mast cells, and eosinophils. They contribute to skin homeostasis by secreting growth factors needed for the survival of keratinocytes, fibroblasts, and endothelial cells. In inflammatory conditions, myeloid cells respond immediately and produce pro-inflammatory mediators that drive the activation of cells in the local vicinity and infiltration of the affected site by peripheral immune cells (Zaba L. Et al., 2009).

The skin also harbours different types of lymphoid cells all of which are important in both steady state and inflammatory responses. Both human and murine skin contain γδ T lymphocytes and αβ T lymphocytes, along with natural killer T cells. γδ T cells are the dominant T cell population in murine skin, while αβ T cells are the dominant T cell population in human skin (Bos J. et al., 1987). αβ T cells such as Tregs and T-helper cells secrete cytokines in response to infection, tissue damage and tumors (Takashi N., 2014). While most αβ T cells undergo apoptosis after a pathogen has cleared, a population of αβ T cells become long-lived memory T cells and reside in the skin. These memory T cells are involved in inflammation upon viral infection by secreting cytokines (Duhen T., 2009).

γδ T cells play key roles in maintaining skin integrity and protecting from malignancy. γδ T cells monitor skin integrity by recognizing damaged cells and producing IGF-1. Activated skin-resident T cells improve the rate of wound closure in cultured human skin in an IGF-1-dependent manner (Toulon A. et al.,

2009). In addition, human dermal γδ T cells express the NKG2D receptor, which stimulates cell lysis (Bauer S., 1999) and they can clones exert cytotoxic responses also against melanoma cell lines (Bachelez H. et al., 1992).

2.2 Non-immune skin cells: Keratinocytes as sensor and central player in the immune defense

Keratinocytes are the main constituents in the epidermis and they orchestrate immune responses if microbes and their molecules penetrate the *stratum corneum* upon mechanical or pathological barrier defects. Keratinocytes express several pattern recognition receptors (PRRs), which contribute to the initial sensing of microorganisms and intracellular signal transduction: Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) (Pasparakis M. et al., 2014). TLRs are the best characterized human PRRs; they recognize conserved microbial structures such as lipopolysaccaride (LPS), lipopeptides, peptidoglycan, flagellin or nucleic acids. Epidermal keratinocytes express the cell surface associated TLRs 1, 2, 4, 5 and 6 and the endosomal TLRs 3 and 9 (Miller L. and Modlin R., 2007). The NOD receptors 1 and 2 are also expressed by human keratinocytes and are intracellular receptors that respond to bacterial peptidoglycan fragments. NOD2 responds mainly to peptidoglycan from Gram-positive bacteria such as S. aureus. NOD2 activation in the presence of TLR signals is especially effective in inducing an inflammatory response (Roth S. et al., 2014).

Keratinocytes are in constant interaction with local immune cells and produce factors crucial in homeostasis and in tissue repair. Keratinocytes communicate with the rest of the immune system through:

- Antimicrobial peptides (cathelicidins and β-defensins) (Afshar M. and Gallo R., 2013)
- Signalling cytokines (e.g. IL-1β) and chemokines which attract other immune cells to the epidermis (Uchi H. et al., 2000)

- Direct activation of primed T lymphocytes and NK cells through major histocompatibility complex I (Black et al, 2007).

2.3 Epidermal cytokines

The correct formation of the epidermal barrier is essential for the barrier function of the skin. This differentiation program relies on well-regulated cell communication processes. Keratinocytes and other skin resident cells produce cytokines that are responsible for the control of cellular communication. Cytokine signaling can result in multiple consequences for the barrier function of the skin. For example, cytokines influence keratinocytes proliferation and differentiation, at least in part by modulating the gene expression program in these cells. One consequence is the expressional control of other cytokines resulting in a complex network of signaling molecules that affect the physiology of keratinocytes and the quality of the skin barrier. Deregulated cytokine expression can thus contribute to dysfunctions of the epidermal barrier as it is observed in many diseases, including atopic dermatitis (AD) and psoriasis (Hanel K. et al., 2013) **(Fig.7)**.

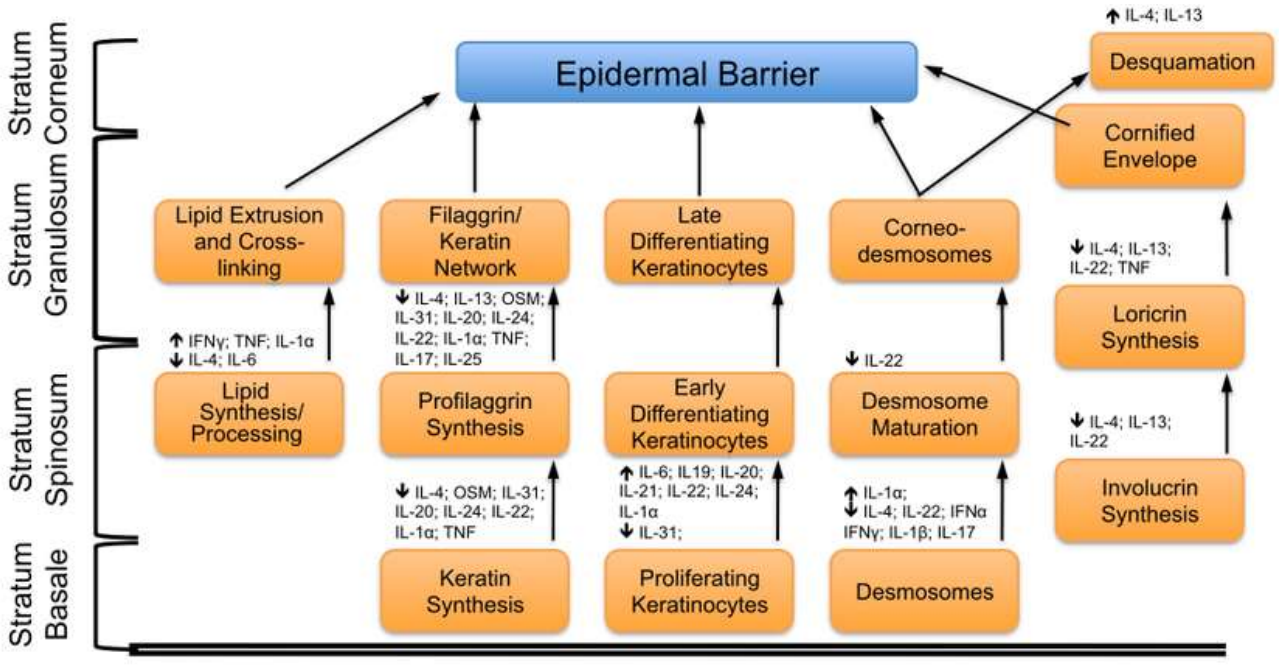

Figure 7 Regulatory effects of the cytokines on the skin barrier formation (Hanel K. et al., 2013).

2.3.1 Epidermal cytokines in wound healing

As mentioned above, the set of epidermal cytokines produced by keratinocytes plays a pivotal role in regulating not only the normal physiological processes of the skin but they also take an active part in response to external insults. An extremely challenging process for the skin is the wound healing. Skin wounds, including acute burns and chronic non-healing ulcers, are commonly observed in clinics. Healing of skin wounds is a complex process, consisting of infiltration of inflammatory cells, cellular proliferation, and tissue remodelling phases, which restore the integrity and functions of the skin. Epithelialization is involved in wound healing through re-establishing an intact keratinocyte layer (Pastar I. et al., 2014). Epidermal stem cells are indispensable for epithelialization and they are regulated by multiple proinflammatory cytokines or growth factors. Upon injury to the skin, human keratinocytes have been shown to express pro- and anti-inflammatory cytokines, along with chemokines and growth factors that contribute to the inflammatory response; the most important pro-inflammatory cytokines produced are: interleukin-1 alpha (IL1α), IL-1β, IL-18, IL-6, IL-8 and tumour necrosis factor-alpha (TNF-α) (Xiao T. et al., 2020).

IL-1α, IL1β, IL-18 and TNFα are termed primary cytokines (Williams R. and Kupper T., 1996) as they are capable of inducing processes that directly result in rapid, local inflammation. Primary cytokines (IL-1 and TNF-α) are able to induce the release of secondary mediators (such as IL-6 and IL-8) in an autocrine or a paracrine manner from other cell types to amplify and assist the inflammatory response. They participate in the inflammation phase of wound healing through activating downstream cascades (Kanji S. and Das H., 2017). They also contribute to the epithelialization phase by mobilizing resident stem/progenitor cells and promoting cell proliferation and differentiation; (Larouche J. et al., 2017).

Among the many growth factors studied in the context of wound healing, transforming growth factor beta (TGF-β) is thought to have the broadest spectrum of effects. TGF-β plays an essential role in wound healing through its pleiotropic effects on cell proliferation and differentiation, extracellular matrix (ECM) production, and immune modulation (Penn J. et al., 2012). The three phases of

wound healing are known as inflammation, tissue formation (proliferation), and maturation (tissue remodelling), which overlap in time. TGF-β plays a critical role in regulating multiple cellular responses that occur in all three phases of wound healing. Inflammation is characterized by the recruitment of immune cells such as neutrophils and macrophages to the injured site in response to chemotactic cytokines such as TGF-β (Wang X. et al., 2006). Once immune cells become activated, they are susceptible to TGF-β1-mediated suppression to reverse the inflammatory process. Thus, TGF-β plays a dual role in the inflammation phase of wound healing by exerting pro-inflammatory effects during the early stages and later contributing to the resolution of inflammation (Finnson K. et al., 2013a; Finnson K. 2013b). Aberrant TGF-β signaling has been implicated in pathological skin disorders, including chronic wounds and excessive scarring (Penn J. et al., 2012).

The high complexity of signaling necessary to coordinate cellular processes participating in wound healing emphasizing the importance of tight spatio-temporal control, in which small changes in levels and timing of any cytokines/growth factor may have a completely different outcome. Alteration in the balance between pro- and anti-inflammatory cytokines is a common feature of chronic non-healing wounds.

2.3.2 Cytokines in cellular senescence: senescence-associated secretory phenotype (SASP)

Cellular senescence occurs in culture and in vivo as a response to excessive extracellular or intracellular stress. The senescence program leads to a cell-cycle arrest preventing the spread of damage to the next cell generation (Campisi J. and d'Adda di Fagagna F., 2007). These cells are found mainly in renewable tissues and in tissues that undergo extended inflammation. Several stresses can trigger cellular senescence: telomere uncapping (termed replicative senescence), mitochondrial deterioration, oxidative stress, severe or irreparable DNA damage (genotoxic stress), and the expression of certain oncogenes (oncogene-induced senescence) (Ben-Porath I. And Weinberg A., 2004). For these reasons, senescence acts as an intrinsic tumor-suppressive mechanism

preventing the propagation of damaged cells. Nevertheless, contrary to common thought, senescent cells are not just arrested in the cell cycle but they are metabolically active and undergo widespread changes in the protein expression and secretion contributing to both tumor suppression and tumor promotion (Faget D. et al., 2019). Indeed, senescent cells impact on tissue microenvironment and on the cell behaviour even in an extrinsic manner by secreting numerous inflammatory, extracellular-modifying and growth factors, collectively referred to as senescence-associated secretory phenotype (SASP). Moreover, has been shown that SASP takes part even in the wound healing by limiting fibrosis and promoting the wound repair through the secretion of growth factors and through the recruitment of immune cells (Demaria M. et al., 2014). The senescent-associated secretory phenotype includes many families of soluble and insoluble factors that can affect surrounding cells by activating several cell-surface receptors and consequently many signal transduction pathways (Coppé J. et al., 2010). SASP factors can be generally divided in three major categories:

- Signaling factors (interleukins, chemokines and growth factors);
- Secreted protease;
- Secreted insoluble proteins/extracellular matrix (ECM) components;

Hence, the SASP factors secretion provide a powerful mechanism through which senescent cells can modify the tissue microenvironment and the cells behaviour. Depending on the biological context SASP factors could have beneficial outcomes (e.g., embryonic development, tumor suppression, wound healing and tissue regeneration) or detrimental ones (e.g., tissue degeneration, chronic inflammation, tumor promotion) (Watanabe S. et al., 2017).

3. Hailey-Hailey Disease (HHD)

Hailey-Hailey disease (HHD, OMIM 16960), also known as familial benign chronic pemphigus, is a rare autosomal dominant genodermatosis with complete penetrance and with an incidence estimated to be 1:50,000-1:40,000 (Engin B. et al, 2015; Burge SM., 1992; Kellermayer R., 2005). Described for the first time in 1939 by the two brothers Hug and Howard Hailey (Hailey H. and Hailey H., 1939), HHD usually appears in the third or fourth decade, although it can occur at any ages (Dobson-Stone C. et al, 2002). Clinically, HHD is characterized by chronic course with remission and recurrence pattern, which may be constant in some patients. The exacerbated skin lesions primarily occur in the sites of friction and intertriginous areas. Heat, sweating, UVB exposure and friction often exacerbates the disease and most patients have worse symptoms during summer (Kellermayer R., 2008) **(Fig.8 a)**.

Histologically, HHD is characterized by loss of cohesion between keratinocytes (acantholysis) with epidermal clefting or vesiculation. Widespread partial loss of the intercellular bridges between keratinocytes gives the epidermis the appearance of a ‘dilapidated brick wall’ (**Fig.8 b-c**). Ultrastructural studies of acantholytic cells in HHD reveal perinuclear aggregates of keratin intermediate filaments that have retracted from desmosomal plaques (Wilgram G., Caulfield J. and Lever W., 1962). Moreover, even if rare, squamous/basal cell carcinomas and melanomas arising in the skin lesions have been described (Chun S. et al., 1988; Cockayne S.E. et al., 2000; Holst V. et al., 2000; Mohr M. et al., 2011).

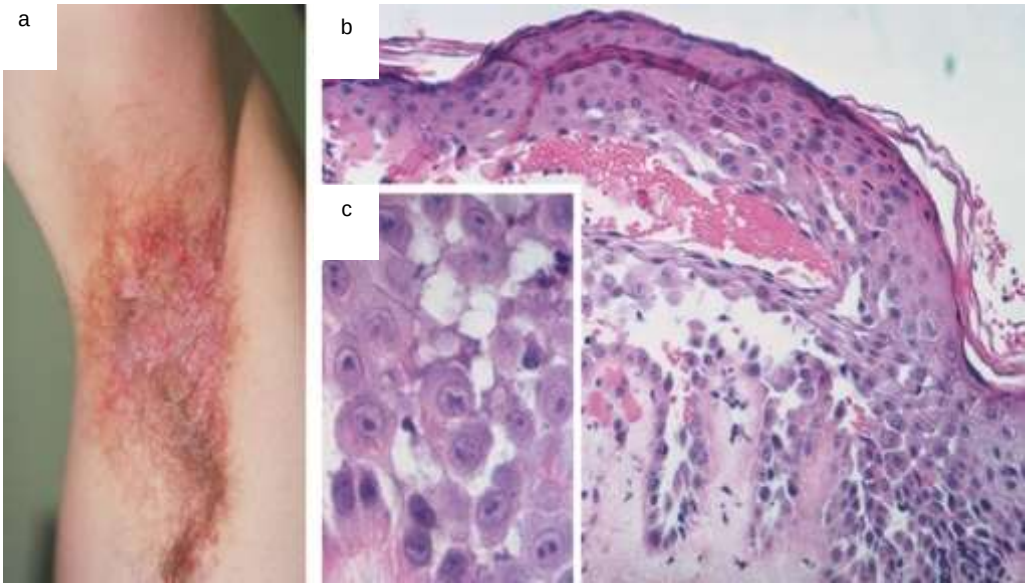

Figure 8: a, Clinical presentation of a patient with HHD with erythema and blisters in axilla. **b**, Histological section of affected skin showing separation of suprabasal cells (acantholysis) (×50). **c**, Higher magnification showing acantholytic cells in the suprabasal layer (×100). (Hu Z et al, 2000)

3.1 The ATP2C1 gene and protein

The genetics and pathophysiology of HHD have been linked to mutations in the *ATP2C1* gene.

The human *ATP2C1* gene is located on chromosome 3q21 and consists of 28 exons (Sudbrak R. et al., 2000; Hu Z. et al., 2000). Alternative splicing at the 3′-end of the human *ATP2C1* pre-mRNA produces four distinct *ATP2C1* variants (corresponding to SPCA1a-d proteins). The ATP2C1 transcript provides instructions for making a protein known as hSPCA1. This protein is an adenosine triphosohate (ATP)-powered Ca2+/Mn2+ pump which uses energy from ATP molecules to pump ions across cell membrane thus regulating the Golgi luminal and cytosolic ionic homeostasis. hSPCA1 consists of five stalk helices in the cytoplasm, ten hydrophobic transmembrana segments and three cytosolic domains (the actuator domain (A), phosphorylation domain (P), nucleotide-binding domain (N)) mostly localized in the Golgi apparatus (Micaroni M. et al., 2016) **(Fig.9)**.

The alternative splicing is not present in other species where we have a single SPCA1.

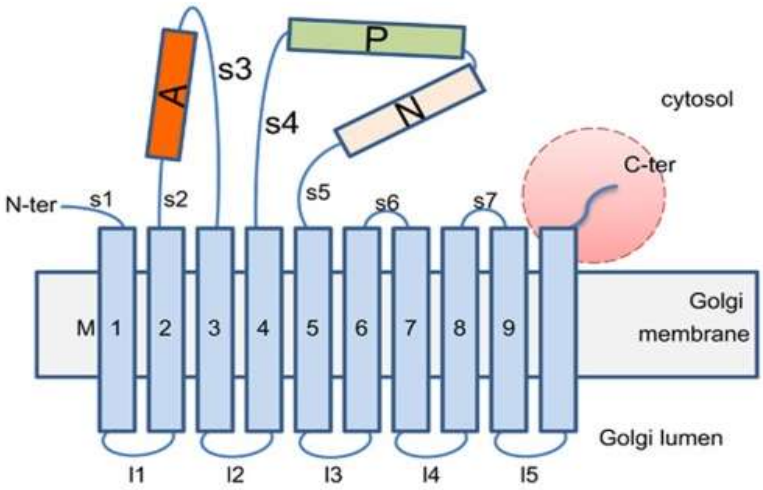

Figure 9 ATP2C1 protein structure. Actuator domain (A), phosphorylation domain (P), nucleotide binding domain (N) and five stalk helices (S) in the cytoplasm and ten transmembrane helices (M). (Adapted from Micaroni M. et al., 2016).

This pump is ubiquitously expressed in human tissues but at higher levels in keratinocytes (Hu Z. et al., 2000).

The relative contribution of hSPCA1 to the total Ca2+ uptake in the Golgi depends on the cell type and it can range from 15% to 50%. However, it has been reported that in keratinocytes the Ca2+ uptake in the Golgi apparatus depends for 67% on hSPCA1 pump activity (Missiaen L. et al., 2007; Callewaert G. et al., 2003)

This finding, in addition to the prominent role played by calcium in the regulation of keratinocytes proliferation/differentiation, may explain, at least in part, why only the skin is affected in HHD, although the exact reason is not yet completely understood.

3.2 Mutations of ATP2C1 gene

To date, at least 177 mutations have been identified on *ATP2C1* gene with no apparent clustering (Micaroni M. et al., 2016).The number and types are as follow: 51 missense mutations (28.8%), 44 small deletions mutations (24.9%), 36

splicing mutations (20.3%), 25 nonsense mutations (14.1%), 12 duplication mutations (6.8%), four complex rearrangements (2.3%), three gross deletions (1.7%) and two small indel mutations (1.1%).The 37.2% of ATP2C1 mutations localize to exons 12, 13, 21, 23, 24, 25. The amino acid sequences encoded from exon 12 and 23 are crucial for Ca2+ binding, from exon 13 for phosphorylation and from exon 21 for Mn2+ binding (Deng H. and Heng X., 2017). Regardless of the type, more than 50% of all ATP2C1 mutations generate premature termination codons (PTCs) leading to mRNA degradation through the nonsense-mediated mRNA decay pathway or to the synthesis of truncated protein with no complete functionality or correct organellar localization. The high proportions of PTCs support the possibility that haploinsufficiency of ATP2C1 is the major mechanism underlying the dominant inheritance of HHD (Micaroni M. et al., 2016).

3.3 The ATP2C1 gene dysfunction and its mechanism in HHD

As previously reported, the proper maintenance of calcium gradient within the epidermis is crucial to regulate proliferation and differentiation processes and consequently the skin barrier function. The Ca2+ gradient in the upper epidermis of HHD is markedly decreased compared to normal skin; this aspect correlate well with the histological main feature of HHD skin that is the acantholysis (Behne M. et al., 2003). Due to hSPCA1 dysfunction, the inefficient increase of external Ca2+ may result in an impairment of both desmosome formation and Ca2+-sensing receptors activation. The latter are responsible for triggering cell-to-cell adhesion, keratinocytes differentiation and reconstituting the calcium gradient (Brini M. and Carafoli E., 2009). The loss of function hSPCA is also responsible for a slower Ca2+ uptake in the Golgi and, at the same time, for a cytosolic calcium concentration increase. Low calcium levels in Golgi may alter crucial post-translational modifications of desmosomes, mainly the glycosylation. On the other hand, the elevation of intracellular calcium levels leads keratinocytes to not respond well to increasing extracellular calcium concentrations. Indeed, HHD keratinocytes display a reduced production of involucrin which makes up the cornified envelope and that is expressed in response to increased extracellular Ca2+ concentrations (Hu Z. et al., 2000; Aberg K. et al., 2007). Moreover, high

cytoplasmic Ca^{2+} levels could overload mitochondria compromising their functionality and leading to redox imbalance (Van Baelen K. et al. 2004).

However, the predominant involvement of skin folds in the cutaneous lesions' manifestation suggests that genetic alterations in *ATP2C1* gene are necessary but not sufficient to lead to the disease onset; other intrinsic and extrinsic factors may also influence the disease manifestation e.g., genetic background, the loss of other Ca2+ regulatory mechanisms, friction, sweating, infections, UV exposure.

3.4 Molecular and immunological features of HHD

Although HHD has been described for the first time in 1939, to date little is still known about the molecular and immunological mechanisms underlying the disorder. As reported, beyond the mutations in *ATP2C1* gene and the consequent deregulation in calcium homeostasis, other factors, referred as "second hits", are needed to trigger the disease onset. The understandings of this aspects are crucial to improve the management of HHD. In the last years, our research activity has been extremely focused on the study of HHD contributing to increase the knowledge about its pathogenesis in particular on molecular level.

One of the first important findings is represented by the evidence that oxidative stress plays a key role in the HHD pathogenesis. Indeed, by employing primary human keratinocytes derived from either normal-appearing skin or cutaneous lesions of patients, Cialfi et al. demonstrated a massive ROS accumulation in the lesion skin derived keratinocytes; this latter aspect was also correlated to a decreased expression levels of Nrf2 and in turn of the detoxifying ability of the cells. Moreover, keratinocytes lesional skin derived displayed a low rate of proliferation leading to a failure of the normal differentiation program (Cialfi S. et al., 2010). In agreement with this observation, the expression of regulatory factors (e.g., Notch1 and its target p63) associated with keratinocytes differentiation and proliferation were extremely altered.

Further investigations, based on RNA-sequencing analysis, pointed out a substantial number of genes differentially expressed in lesioned skin compare to

the normal appearing one. A first group of genes differentially expressed was represented by genes involved in the inflammatory response and in the wound healing process. In particular, the mRNA levels of the proinflammatory cytokines IL-6, IL-1, IL-32-34 and TGFB2 were up-regulated (Cialfi S. et al., 2016). Conversely, IL-33, IL-24 levels are decreased in keratinocytes derived from HHD lesions; IL-33 may play pivotal roles in the maintenance of cutaneous homeostasis and the acceleration of normal wound healing (Yin H. et al., 2013). In skin wounds, interleukin 6 (IL-6), the IL-1 family and transforming growth factor (TGF-β) are crucial cytokines that regulate the re-epithelialization process (Pastar I. et al., 2014; Larouche J. et al., 2017; Finnson K. et al., 2013a-b). Hence, since the success of wound healing process depends on growth factors, cytokines and chemokines (Hanel K. et al., 2013; Xiao T. et al., 2020), alterations in their expression could explain, at least in part, why HHD skin lesions do not heal and show recurrent infections. A better understanding of the influence of deregulated cytokines on ATP2C1-defective keratinocytes proliferation, differentiation and wound repair mechanisms may help to design treatment strategies to improve wound healing and microbial infection regression in HHD patients.

Therefore, these data indicate that HHD keratinocytes are defective in managing both oxidative-stress response and wound signal that ultimately could contribute to the poor healing of HHD lesions.

The transcriptome analysis carried out by Cialfi S. et al. had also revealed a strong down-modulation of DDR genes in HHD skin lesions. It has been proposed that ATP2C1 loss of function leads to an increased production of ROS and to an upregulation of the Notch1 signaling. Notch1 signaling is responsible for the subsequent ATM downregulation and, thus, for the DDR repression. Increased ROS levels and ATM loss would produce DNA damage up to a threshold that keratinocytes cannot repair, which would then promote terminal differentiation; a premature differentiation and the consequent exhaustion of the transit amplifying component of the skin, could result in a compromised capacity of the skin to regenerate itself (Cialfi S. et al., 2016). Moreover, generation of DNA damage as well as the alteration in tissue microenvironment, are important steps in the

process of carcinogenesis. HHD rarely degenerates in skin cancer although squamous cell carcinoma and basal cell carcinoma arising in lesions of HHD have been described in the literature (Chun S. et al., 1988; Cockayne S.E. et al., 2000; Holst V. et al., 2000; Mohr M. et al., 2011).

3.5 Diagnosis and treatment

HHD can be easily mistaken with other skin conditions such as psoriasis, eczema, contact dermatitis, fungal infections and with a similar genodermatosis, called Darier disease. Thus, a skin biopsy and genetic test on *ATP2C1* mutations are necessary to make a proper diagnosis.

To date, there is no long-term treatments known to be effective in HHD patients. The traditional treatments are aimed at symptomatic relief. Since bacterial, fungal and viral superinfections commonly exacerbate HHD skin lesions making them persistent, topical and systemic antibiotics, antifungal and antiviral agents are used. In addition, in order to diminish inflammation and the pricking/itchy sensations, topical and systemic corticosteroid are administrated to patients.

However, prolonged treatment course of steroids is limited due to their side effects, most commonly skin atrophy. This last aspect must be carefully considered, because in HHD-patients, lesion development is associated with the simple friction of the skin. Additionally, patients develop lesions refractory to corticosteroids. As lesions became recalcitrant to Standard of Care (SOC) treatment, several possible treatments have been proposed including: Botulinum toxin injection, to reduce sweat production and the skin maceration, photodynamic therapy (Arora H. et al., 2016), diseased skin dermabrasion (Hamm H., 1994), as well as topical immunomodulators administration to reduce pro-inflammatory cytokines production (Tchernev G. and Cardoso J.C., 2011).

As described, our research group found that the increase of oxidative stress plays a central role in pathogenesis of HHD (Manca S. et al., 2011; Cialfi S. et al., 2010). In this regard, Biolcati G. et al. found that Nle4-D-Phe7-a-melanocyte-stimulating hormone (afamelanotide), an a-MSH analogue with antioxidant proprieties, has a therapeutic efficacy in patients with treatment-resistant HHD. Afamelanotide is able to partially restore Nrf2 expression and the defective

proliferative capability of lesion- derived keratinocytes *ex vivo*. Moreover, a clinical trial performed on this compound administered by injection once a month showed improvements in 30 days after first injection and complete clearance of HHD lesions after 60 days, independently of the lesion location (Biolcati G. et al., 2014).

However, since the evidence for the above indicated treatments of HHD is limited to case reports, case series, and expert opinion, the development of causal treatment strategies (i.e., molecular therapy-based) is highly desirable and could be reached through intensified efforts to elucidate the various molecular mechanisms underlying the disorder.

3.6 Animal model in HHD study

Research on molecular pathways, treatments or management strategies for rare diseases can be challenging primarily due to the limited number of individuals who will be eligible to participate in any given study. In addition, ethical and psychological aspects must be carefully considered when doing molecular and/or clinical research on patients with rare disease. Obviously, this is also true for the HHD.

Working *ex vivo* by using keratinocytes derived from normal and lesioned skin of HHD patients is not always possible. Punch biopsies in HHD skin can trigger the manifestation of new skin lesions or exacerbate the existing ones dramatically worsening the clinical and psychological conditions of patients. Another issue making the molecular research on HHD even more difficult is the inability to use mouse models because of the poor recapitulation of the main human pathological features. In fact, while null mutant Spca1−/− embryos do not survive to the gestation due to defects in neural tube closure, heterozygous mice for ATP2C1 do not develop the typical vesicular lesions of human patients and show an increase susceptibility to squamous cell tumors, a phenotype rarely observed in humans with HHD (Okunade G. et al., 2007).

In the last 10-20 years the yeast, in particular *S. cerevisiae*, has become increasingly used in biomedical research as an effective and simple model

system to understand the molecular players associated to a given pathology. Indeed, about 30% of the genes known to be involved in human diseases have a yeast orthologs (Foury F., 1997).

This is still true in the study of Hailey-Hailey disease. Both the budding yeasts *S. cerevisiae* and *Kluyveromyces lactis* (*K. lactis*) express the orthologue gene of ATP2C1, called *PMR1* (plasma membrane ATPase related) (Uccelletti D. et al., 1999; Rudolph H.K. et al., 1989). Indeed, the expression of the human ATP2C1 in yeast fully rescues the yeast *pmr1* ipersensitivity to Ca2+ chelators and Mn2+ toxicity (Ton V. et al, 2002). Cells deprived of *Pmr1p* display pleiotropic phenotypes, some of them resembling those reported in HHD keratinocytes, including alterations in Ca2+ homeostasis, mitochondrial dysfunctions and increased production of reactive oxygen species (Hu Z. et al. 2000; Uccelletti D. et al., 2005). This latter aspect is associated with the decreased action of some detoxifying systems both in yeast cells and in the lesional-derived keratinocytes of HHD patients (Ficociello G. et al., 2016).

II. AIMS OF THE WORK

While a strong relationship exists between mutations in *ATP2C1* gene and Hailey-Hailey disease development, the precise mechanism through which mutations induce skin lesions is unknown and, to date, there is no treatment known to be effective and side effects free. In the last years, our research activity has highlighted how the disease is closely connected with defects in managing oxidative-stress response, re-epithelialization process as well as DNA damage response contributing to the poor healing of HHD lesions (Cialfi S et al., 2010; Manca S. et al., 2011; Biolcati G. et al., 2013; Ficociello G. et al., 2016; Cialfi S. et al., 2016).

During the PhD course, my research activity has, therefore, focused on the following aims.

1: Identification of compounds with a potential therapeutic effect in Hailey Hailey disease.

The lack of effective and safe long-term treatment and the recent discoveries about some of the molecular mechanisms underlying the disease, has prompted us to search for compounds that, acting on such altered mechanisms, may be used for the development of safer and more specific/effective therapy. All the data obtained were included in two different papers (**Zonfrilli A. et al., 2018; Cialfi S. et al., 2019**) inserted in the following “RESULTS” section of this PhD thesis (Results: 1.1 e 1.2).

2: Gaining insight into the role of Notch1 signaling in the response to genotoxic stress

Generation of DNA damage as well as the alteration in tissue microenvironment, are important steps in the process of carcinogenesis. HHD rarely degenerates in skin cancer although squamous cell carcinoma and basal cell carcinoma arising in lesions of HHD have been described in the literature (Chun S. et al., 1988; Cockayne S.E. et al., 2000; Holst V. et al., 2000; Mohr M. et al., 2011).

As previously reported, ATP2C1 defective keratinocytes are characterized by a persistent repression of DNA Damage response genes caused by ATM down-modulation that in turn is Notch1 dependent (Cialfi S. et al., 2016).

Basing on the evidence that ATM and consequently the DDR response are major targets of oxidative stress-induced Noch1 activation, during my PhD dissertation I investigated how Notch1 is mechanistically implicated in the response to genotoxic stress and thus in the DDR function. All the data obtained were included in a recent published paper (**Zonfrilli A. et al., 2019**) inserted in the following “RESULTS” section of this PhD thesis (Results:2).

III. RESULTS

1.1 Yeast-Based Screen to Identify Natural Compounds with a Potential Therapeutic Effect in Hailey-Hailey Disease (Zonfrilli A. et al., 2018).

1.2 Hypotonic, Acidic Oxidizing Solution Containing Hypochlorous Acid (HClO) as a Potential Treatment of Hailey-Hailey Disease (Cialfi S. et al., 2019).

Given the impossibility to use mouse model, we set up a yeast-based screening assay in order to identify natural compounds able to rescue or ameliorate phenotypes of *K. lactis pmr1*Δ cells. Due to the great relevance of the oxidative stress in HHD-derived keratinocytes, we first of all evaluated if the drugs were able to recover the oxidative-stress alterations of yeast mutant. From the first screening, we selected six compounds that were utilized for further analysis. Specifically, we analyzed if the six positive hits were able to alleviate other main defects of *pmr1*Δ cells, like the calcium homeostasis alteration, the cell wall defects and the mitochondrial dysfunction. All those compounds active in yeast were then tested on our *in vitro* model of HHD. We provided the evidence of the selected compounds efficacy also on human ATP2C1-defective keratinocytes. In particular, the selected compounds improved keratinocytes proliferation rate, restored the Nrf2 expression and, in turn, the cellular antioxidant response. Furthermore, one compound, hypochlorous acid, was also able to affect the deregulated cytokines expression by differentially modulate TGFB1 and TGFB2 levels.

Our results validate the use of the yeast *K. lactis* to screen drugs with potential therapeutic effect in HHD disease treatment bypassing, at least in part, the difficulties related to the absence of mouse model.

2. PLK1 Targets NOTCH1 during DNA Damage and Mitotic Progression (Zonfrilli A. et al., 2019).

Induction of DDR and of cell cycle arrest in response to DNA damage represents a protective mechanism against harmful mutations. In this work, we revealed the existence of an interplay between the proteins Polo-like kinase 1 (PLK1) and Notch1 and, furthermore, we described how this interaction is involved in the regulation of the G2 phase of the cell cycle. Interestingly, we found that the interaction between Notch1 and PLK1 is functionally important also during the DNA damage response, as we found that whereas PLK1 activity is inhibited, Notch1 expression is maintained when cells in G2 are challenged with DNA damaging agents. In particular, we found that Notch1 signaling protects immortalized HaCaT cells from DNA damage-induced apoptosis and furthermore, we demonstrated that epithelia cancer cells, growth-arrested after DNA damage, use the induction of Notch1 signaling to develop a secretory phenotype and thus to promote an inflammatory cytokines secretion. Indeed, when we treat SCCO22 cells with DNA damaging drugs, e.g., doxorubicin, we detected an increase of IL-6 and IL-8 that is Notch1 dependent.

In summary, in order to get a better understanding of how Notch1 is involved in the DDR, we identified a novel mechanism through which Notch1 takes part in the genotoxic stress response.

International Journal of
Molecular Sciences

MDPI

Article

Yeast-Based Screen to Identify Natural Compounds with a Potential Therapeutic Effect in Hailey-Hailey Disease

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Received: 27 April 2018; Accepted: 13 June 2018; Published: 20 June 2018

Abstract: The term orthodisease defines human disorders in which the pathogenic gene has orthologs in model organism genomes. Yeasts have been instrumental for gaining insights into the molecular basis of many human disorders, particularly those resulting from impaired cellular metabolism. We and others have used yeasts as a model system to study the molecular basis of Hailey-Hailey disease (HHD), a human blistering skin disorder caused by haploinsufficiency of the gene *ATP2C1* the orthologous of the yeast gene *PMR1*. We observed that *K. lactis* cells defective for *PMR1* gene share several biological similarities with HHD derived keratinocytes. Based on the conservation of ATP2C1/PMR1 function from yeast to human, here we used a yeast-based assay to screen for molecules able to influence the pleiotropy associated with *PMR1* deletion. We identified six compounds, Kaempferol, Indirubin, Lappaconite, Cyclocytidine, Azomycin and Nalidixic Acid that induced different major shape phenotypes in *K. lactis*. These include mitochondrial and the cell-wall morphology-related phenotypes. Interestingly, a secondary assay in mammalian cells confirmed activity for Kaempferol. Indeed, this compound was also active on human keratinocytes depleted of ATP2C1 function by siRNA-treatment used as an in-vitro model of HHD. We found that Kaempferol was a potent NRF2 regulator, strongly inducing its expression and its downstream target *NQO1*. In addition, Kaempferol could decrease oxidative stress of ATP2C1 defective keratinocytes, characterized by reduced NRF2-expression. Our results indicated that the activation of these pathways might provide protection to the HHD-skin cells. As oxidative stress plays pivotal roles in promoting the skin lesions of Hailey-Hailey, the NRF2 pathway could be a viable therapeutic target for HHD.

Keywords: Hailey-Hailey; NRF2; NOTCH1; oxidative-stress

1. Introduction

Hailey-Hailey disease (HHD), also called benign familial pemphigus, is an autosomal dominant blistering skin disorder, manifesting in the 3rd to 4th decades of life. The overall incidence and prevalence of HHD is unknown, although some authors have reported an incidence between 1:40,000 and 1:50,000 [1,2]. The genetics and pathophysiology of this skin disorder have been linked to mutations in the *ATP2C1* gene [3,4]. The gene, located on the long arm of chromosome 3, 3q21-q24 region, encodes the human secretory pathway Ca^{2+}/Mn^{2+} ATPase, hSPCA1 [5]. Although ATP2C1

is mostly localized to the Golgi apparatus, it regulates also endoplasmic reticulum (ER) Ca^{2+} stores with effects on both Golgi and ER functions. The lack of ATP2C1 in keratinocytes leads to the loss of cell-to-cell adhesion (acantholysis) among the cells of the suprabasal layer of epidermis probably due to a retraction of keratin intermediate filaments from the desmosomal plaques [6]. Although *ATP2C1* mutations are 100% penetrant, currently there is no treatment known to be effective in reducing the cutaneous manifestations of HHD. The Standard of Care (SOC) treatment consists in either topical or oral administration of corticosteroids often used in combination with topical/systemic antimicrobial agents. However, prolonged treatment course of steroids is limited due to their side effects, most commonly skin atrophy. This last aspect must be carefully considered, because in HHD-patients, lesion development is associated with the simple friction of the skin, and we found that HHD-keratinocytes are characterized by wound defects [7]. Additionally, patients develop lesions refractory to corticosteroids. As lesions became recalcitrant to SOC treatment, several possible treatments have been proposed, including: Botulinum toxin injection and photodynamic therapy [8]. However, evidence for the above indicated treatments of HHD is limited to case reports, case series, and expert opinion. The development of causal treatment strategies (i.e., molecular therapy-based) is highly desirable and could be reached through intensified efforts to elucidate the various molecular mechanisms underlying the disorder. HHD is associated with the loss of a single copy of the *ATP2C1* gene. *ATP2C1* is likely essential in humans, as more severe phenotypes are found in patients who suffer clonal loss of both copies of the gene [9]. Consistently, mice embryos homozygous for null mutations in *ATP2C1* die with defects in neural tube closure, while heterozygotes show susceptibility to squamous cell tumors, a phenotype observed rarely in humans with Hailey-Hailey; [10,11] and our personal observation); however, this mouse model fails to reproduce the clinical manifestation of the disease, unfortunately opposing the applicability of this mouse model in HHD. Yeast has been increasingly used as a model and tool for biomedical research [12,13], based on the observation that basic cellular functions are conserved from yeast to humans and that disease's key players are often evolutionarily conserved. Indeed, about 30% of the genes known to be involved in human diseases have a yeast ortholog [14,15]. For these reasons, this simple organism is widely used for high-throughput genetic and small-molecule screens to find possible pharmacological drugs for many human diseases. This is still true in the study of Hailey-Hailey disease. Indeed, both the budding yeasts *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Kluyveromyces lactis* (*K. lactis*) express the orthologous gene of *ATP2C1*, *PMR1* (plasma membrane ATPase related) [16–18]. Yeast cells deprived of *PMR1* display pleiotropic phenotypes; some of them have been reported also for HHD keratinocytes, including alterations in Ca^{2+} homeostasis, mitochondrial dysfunctions and an increased production of reactive oxygen species (ROS) [3,19,20]. Oxidative stress represents a hallmark of the keratinocytes derived from the lesions of HHD patients and it could be associated to the decreased action of some detoxifying systems. Particularly, we previously demonstrated that one of the detoxifying enzymes involved in the pathophysiology of HHD is the Glutathione S- transferase (GST) [21]. Indeed, performing a genetic screening, we found that the expression of mammalian GST in the yeast *K. lactis* lacking *PMR1* recovers the oxidative alterations of mutant cells, promoting a reduction to the sensitivity to ROS generating compound (H_2O_2), decreasing its cellular content and restoring the mitochondrial function. Additionally, we showed that, both in yeast cells and in the lesional-derived keratinocytes of HHD patients, the expression of this detoxifying gene is down-regulated [21]. Based on these observations, in this study we establish a yeast-based screening assay, designed to identify drugs that could be active against Hailey-Hailey disorder. Natural product collections are bioactive and structurally diverse molecules. It has been estimated that 60% of current FDA-approved drugs have origins in natural products, illustrating the power of these compounds in drug discovery [22]. Thus, we took advantage of a library of 131 natural compounds to analyze their ability to suppress the phenotypes of *K. lactis pmr1*Δ cells. Due to the great relevance of the oxidative stress in HHD-derived keratinocytes, in the initial screening system we evaluated if the drugs were able to recover the oxidative-stress alterations of our mutant. With this aim, we analyzed the growth

in the presence of H_2O_2 or menadione, two generators of ROS at extracellular and mitochondrial level, respectively. From the first screening, we selected six compounds that were utilized for further analysis. Specifically, we analyzed if the six positive hits were able to alleviate other main defects of *pmr1*Δ cells, like the calcium homeostasis alteration, the cell wall defects and the mitochondrial dysfunction. Moreover, we showed that one of the identified hit in the yeast screening was effective also in cellular culture of keratinocytes silenced for ATP2C1. These results validate our approach that provides the use of the yeast *K. lactis* to screen drugs with potential to treat the HHD disease.

2. Results

2.1. Primary Screen of Chemical Libraries Using KLPMR1-Based Assay

Previously, we demonstrated the feasibility of using the yeast Kluyveromyces lactis for modeling Hailey-Hailey disease [21]. In the present study we performed a pharmacological screening using a library of 131 natural molecules to analyze their ability to suppress the *pmr1*Δ phenotypes (Figure 1A). The drug collection includes inhibitors, activators and antagonists acting on molecular targets involved in different signaling pathways. Since the lack of the Golgi Ca^{2+}-ATPase in yeast, as well as in human HHD keratinocytes, induced a prominent increase of ROS production [20,23], we started our screening testing with the natural molecules capability to ameliorate the growth properties of the *pmr1*Δ strain under oxidative-stress conditions. Toward this aim, ROS conditions were achieved either exogenously by H_2O_2 administration or endogenously by menadione treatment (Figure 1B). First, we tested all the molecules at a concentration of 200 M. The compounds that had partial or no effects were tested at a concentration of 250 M. Furthermore, the molecules found to be toxic at 200 M were analyzed at a lower concentration (100, 10 and 5 M) and those compounds showing either toxicity or ineffectiveness at a lower concentration were excluded from further analysis (Table S1). As shown in Figure 1B, six compounds were able to reduce the sensitivity of *klpmr1*Δ mutant to menadione and/or H_2O_2. The molecules S2328, S2386, S2314 and, more effectively, the S2387 and S2267 decreased the growth defects of mutant cells in the presence of menadione. Meanwhile, S1973 and S2314 were more effective against H_2O_2 (Figure 1B). This indicates that the action of the different compounds depends on the localization of the ROS source. These six molecules, selected from the preliminary screen, belong to different class of drugs. Indeed, the S2386 and S1973 (Indirubin and Cyclocytidine) are used in medicine as chemotherapeutics [24,25], S2387 and S2314 (Lappaconite Hydrobromide and Kaempferol) have an anti-inflammatory action [26], and S2328 and S2267 (Nalidixic acid and Azomycin) are mainly recognized as anti-bacterial drugs [27]. A dose–response curve was then performed for the six compounds (Figure S1) and the EC50 was determined as reported in Table 1.

Table 1. Median effective concentration (EC50) of the compounds selected in the yeast primary screening.

Chemicals	Oxidative Stress Selection	EC50 (mM)
S1973 (Cyclocytidine)	H_2O_2	51.26 ± 1.19
S2267 (Azomycin)	Menadione	102.64 ± 0.50
S2314 (Kaempferol)	H_2O_2	50.5 ± 0.44
S2328 (Nalidixic acid)	Menadione	50.13 ± 4.84
S2386 (Indirubin)	Menadione	48.56 ± 3.01
S2387 (Lappaconite)	Menadione	9.35 ± 0.42

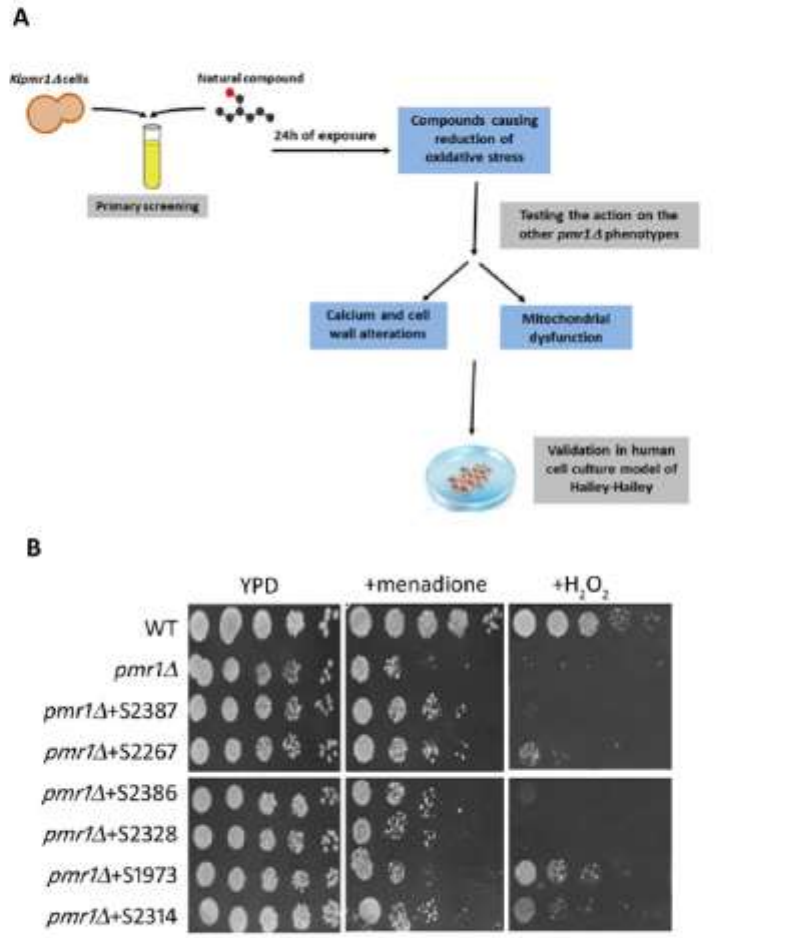

Figure 1. (**A**) General flowchart of the natural products screen approach. The primary screening is performed to identify compounds that alleviate the oxidative stress of *pmr1*-mutant cells. Then the effects of the positive hits are tested for the other phenotypes of the mutant strain. The final step is to test the selected molecules on the human cell cultures used as model for Hailey-Hailey disease; (**B**) The *PMR1*-deleted strain exposed or not for 24 h to different natural products was tested for its ability to grow with or without the 60 μM menadione or 4 mM H_2O_2. Wild type cells (WT) were used as control.

2.2. Yeast-Hits Rescue Multiple Defects in *pmr1*Δ Cells

Our next goal was to assess if the hits selected in our screening were also able to rescue the multiple phenotypes associated with the deletion of the *PMR1* gene. As reported by [20], the deletion of the *PMR1* in *K. lactis* cells led to a higher content of intracellular calcium as well as growth defects when the homeostasis of this ion is disrupted by EGTA. For this aim, we tested the sensitivity of the *Klpmr1*Δ cells treated with the selected hits to the calcium chelator EGTA. As indicated in Figure 2, only two compounds had a positive effect: S2386 and S1973, meanwhile the other four molecules were ineffective.

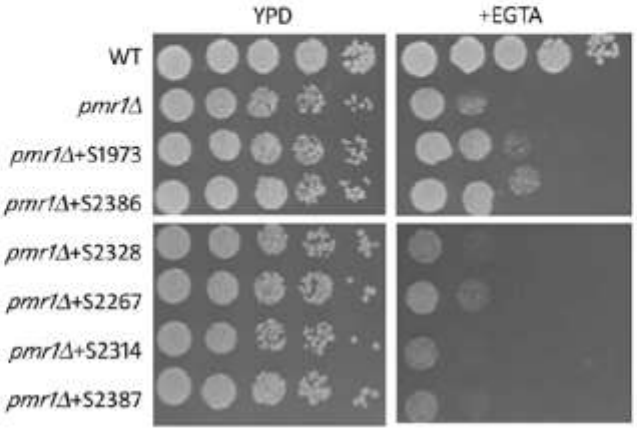

Figure 2. Analysis of calcium alteration. WT and *Klpmr1*Δ cells exposed or not to the individual natural molecules, were grown for 24 h in Yeast Extract-Peptone-Dextrose (YPD) medium at 30 °C. Then, serial dilutions of the cultures were spotted onto solid medium supplemented or not with 20 mM EGTA. Scale bar:

2.3. Cell Wall Phenotype

K. lactis strain deleted for *PMR1* gene had defects in the cell wall organization. Indeed, as shown by using the fluorescent dye Calcofluor white (CFW), which binds the cell wall component chitin, we previously reported that in wild type cells the chitin is mainly deposited to the bud-emergence sites, whereas in the *Klpmr1*Δ strain the fluorescence is distributed across the entire cell wall [17]. To analyze the effect of the selected hits on the cell wall structure of our mutant, the CFW staining was performed. We found that all the six molecules were able to recover the wild type-like chitin distribution (Figure 3). However, the molecules S1973 and S2314 induced a recovery of 40%; meanwhile, the compounds S2267, S2387 and S2386 relieved the wall disorganization in about 50% of the cells. Particularly, the compound S2328 strongly recovered the cell wall morphology in 80% of *klpmr1*Δ cells.

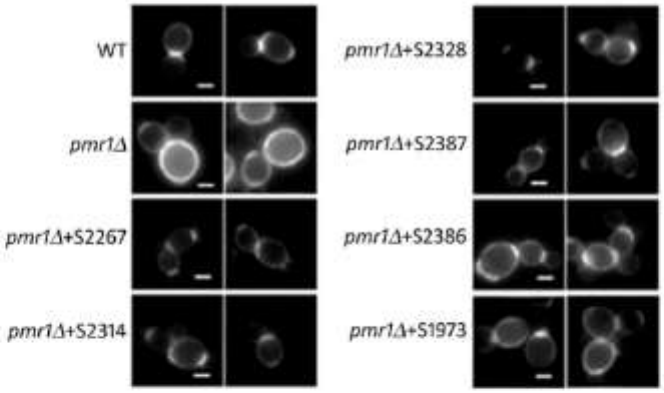

Figure 3. Chitin distribution of mutant cells treated with the six selected products. *PMR1*-deleted cells, grown with or without the individual compounds for 24 h at 30 °C, were stained with the chitin-binding dye CFW. At least 500 cells were analyzed for each treatment to determine the percentage of cell wall recovery. Wild type cells (WT) represent the positive control. Scale bar 2 μm.

2.4. Mitochondrial Morphology

Mitochondria are responsible for the main source of ROS in most cells, linking mitochondrial respiration with ROS effects on cellular function [28]. Wild type cells show a tubular network of mitochondria as long as in the *Klpmr1*Δ strain these organelles appear as dots, indicating an alteration in their functionality [20]. Thus, we addressed the capability of the selected hits to rescue the mitochondria alteration of *Klpmr1*Δ cells. With this aim, we incubated our sample with the fluorescent probe DASPMI that is taken up by mitochondria as a function of membrane electrochemical potential. As shown in Figure 4, we found that three drugs (S2386, S2314 and S2387) restored the wild type-like tubular network of mitochondria. The drug S2314 totally relieved the mitochondria defects of our mutant while the molecules S2386 and S2387 worked in the 60% of *Klpmr1*Δ cells. Overall, our data indicate that each molecule acts on specific phenotypes of *Klpmr1*Δ cells (Table S2). This is in agreement with the fact that the selected molecules belong to different classes of drugs.

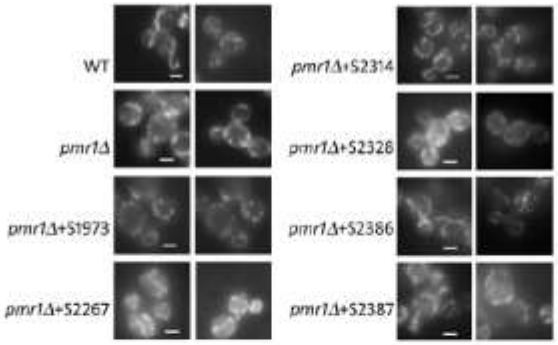

Figure 4. Effect of the natural compounds on the altered mitochondrial function of *pmr1*Δ cells. The mutant cells, untreated or treated with the indicated molecules for 24 h, were stained with the vital dye DASPMI and immediately the fluorescence micrographs were taken. To calculate the percentage of cells with altered tubular mitochondria morphology, at least 500 cells were analyzed for each condition. Wild type strain (WT) was used as a control. Scale bar 2 μm.

2.5. Drugs Active in the Yeast-Based Assay Were Also Active in Human Cells

We next tested the compounds that were active in our yeast-based assay in a human cell-based model of HHD-disease; in particular, we used a siRNA-ATP2C1 to mimic *ATP2C1*-loss of function [7,23,29]. We previously found that siATP2C1-treated cells share most of the defects observed in *K. lactis* cells defective for *PMR1* gene including oxidative stress [7,21,23,29,30]. We established that ATP2C1 inhibition in both immortalized and primary keratinocyte cells results in an impaired proliferation; thus, the compounds from the yeast screening were tested in human keratinocytes and we sought to perform our primary screen in HaCaT cells determining how the morphology/cell proliferation defects of si-ATP2C1 cells were influenced by the treatment with the identified compounds. HaCaT cells were transfected with either siATP2C1 or -siCTR after 24 h treated with the indicated compound for further 24 h. Interestingly, we observed that Kaempferol (EC50 0.8 μM) treatment rescued the aberrant cell morphology and cell growth ability of siATP2C1-treated cells (Figure 5). In a second step, we validated the Kaempferol effect by performing a secondary analysis in human primary keratinocytes. Similarly, to our observation in HaCaT cells, we observed that Kaempferol treatment was able to rescue the aberrant cell morphology/growth of siATP2C1 treated human primary keratinocytes (Figure 6).

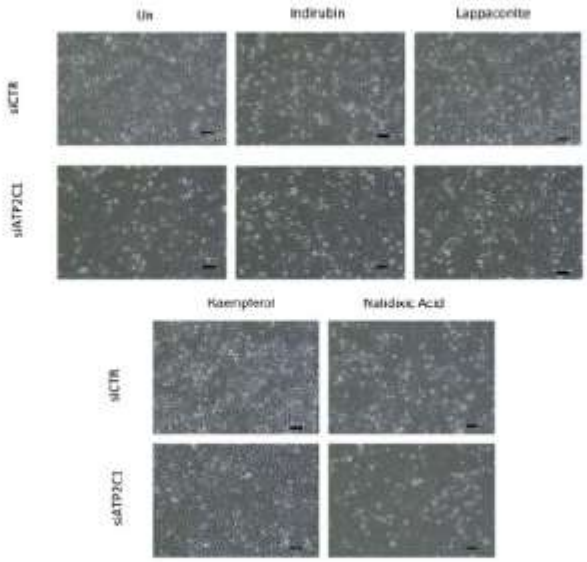

Figure 5. Keratinocytes-derived cell line, HaCaT, was transfected with either siRNA-CTR or siRNA-ATP2C1; 24 h post-transfection, cells were treated with the indicated compounds at 10 μM for a further 24 h and analyzed by microscopy. (100× magnification). The potencies (EC_{50} = 0.8 μM +/− 0.1) of Kaempferol were obtained from the dose–response curves using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Scale bar: 50 μm.

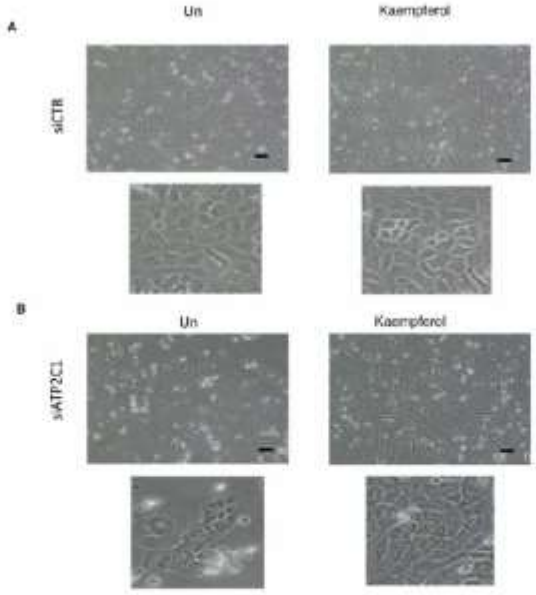

Figure 6. (**A**,**B**) NHKCs (primary human keratinocytes) were transfected with control (siRNA-CTR) or ATP2C1-specific siRNA oligonucleotides; 24 h later, cells were treated with Kaempferol (10 μM) for 24 h and analyzed by microscopy. (100× magnification). Each of the lower images is an enlarged subset of the image above. Scale bar: 50 μm.

2.6. Potential Mechanism of Kaempferol against ATP2C1-Induced Oxidative-Stress through Regulation of Nuclear Factor Erythroid-2-Related Factor 2 Signaling

HHD lesion-derived keratinocytes are characterized by increased oxidative stress and decreased expression levels of both NOTCH1 and NRF2 [7]. We have established that altered function of these factors plays an important role in the alteration observed in HHD-derived keratinocytes [7]. Both NOTCH1 and NRF2 factors are important determinant of skin homeostasis and we found that they are differentially regulated between normal and HHD-derived keratinocytes, as well as in HaCaT cells interfered for ATP2C1 function [7]. Thus, we tested the expression of these two factors in response to Kaempferol treatment. Both HaCaT cells and primary human keratinocytes were transfected with either siATP2C1 or -siCTR, and 24 h post-transfection, they were treated with Kaempferol for a further 24 h. In both cell types NOTCH1 expression wasn't affect by Kaempferol treatment, while NRF2 expression was strongly increased. This observation indicates that loss of NRF2 activity in defective *ATP2C1*-cells might have a direct effect on increased oxidative-stress (Figure 7). Thus, the antioxidant property of Kaempferol and its ability to restore NRF2 expression might play a role in reducing the oxidative-stress of siATP2C1-treated cells. Interestingly, Kaempferol did not significantly affect the steady-state level of *NRF2* mRNA, indicating that it stimulates NRF2 expression by protein stabilization (Figure 7). NRF2 activation directly regulates antioxidant gene transcription [31,32]. NRF2 activation can be modulated by flavonoid as Kaempferol [31,32]; thus, the reduced expression NRF2 in lesioned HHD skin may play a role in the transcriptional down-regulation of antioxidant genes [7,21,30]. Therefore, we first tested if Kaempferol treatment affects the level of oxidative-stress present in siATP2C1-treated cells (Figure 7F). The percentage of DFCA-positive cells in siATP2C1 cells reached 40–60% at 48 h after transfection, whereas only 15% of the siRNA-CTR control cells were DFCA-positive (data not shown and [7]). Interestingly, treatment with Kaempeferol reduced the oxidative stress of both siCTR and of siATP2C1-treated keratinocytes. However, the extent of oxidative-stress in siATP2C1 still remained higher than siCTR cells, indicating that the causative factors and underlying mechanism of oxidative stress still remain active (Figure 7F). NRF2 directly affects the homeostasis of ROS by regulating the expression of several antioxidant genes. Therefore, we analyzed the expression of NRF2-target genes in siATP2C1 and si-CTR-interfered primary keratinocytes after Kaempferol treatment. In ATP2C1 defective cells we observed the loss of NRF2 protein expression; however, only two down-regulated genes (*NQO1* and *GST-M1*) were similarly altered by siRNA-ATP2C1 treatment (Figure 8). Kaempferol treatment partially suppressed oxidative stress in ATP2C1-defective cells and this was paralleled by increased levels of NRF2 and *NQO1/GST-M1* expression. Our data indicate that Kaempferol treatment rescues the impaired NRF2 expression of ATP2C1 defective cells and, in turn, *NQO1/GST-M1* expression (Figure 8).

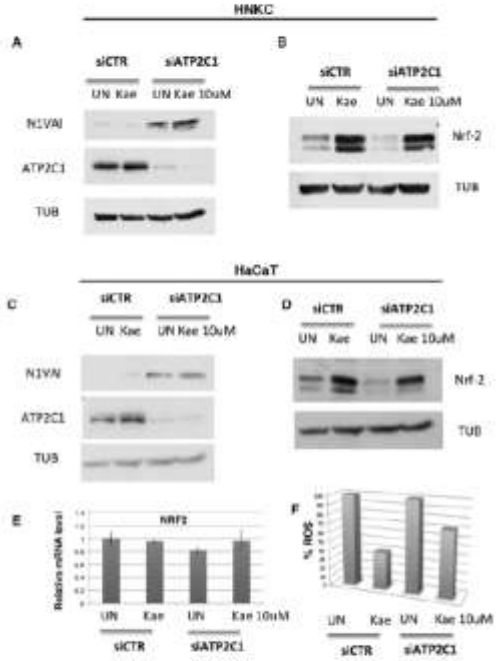

Figure 7. Cell extracts were prepared from both NHKCs (**A**,**B**) and HaCaT cells (**C**,**D**) transfected with either control (siRNA-CTR) or ATP2C1-specific siRNA oligonucleotides; 24 h later, cells were treated with Kaempferol (10 μM) for 24 h and the cell extracts analyzed by western blot; (**E**) Cells were treated as in C, and expression of NRF2 was determined by RT-PCR; (**F**) Keratinocytes-derived cell line, HaCaT, was transfected with either siRNA-CTR or siRNA-ATP2C1 and cells were analyzed by flow cytometry. The percentage of ROS-positive cells is also shown. The absolute value of ROS of both from siRNA-CTR and siRNA-ATP2C1 Kaempferol-untreated cells was arbitrary indicated as 100%.

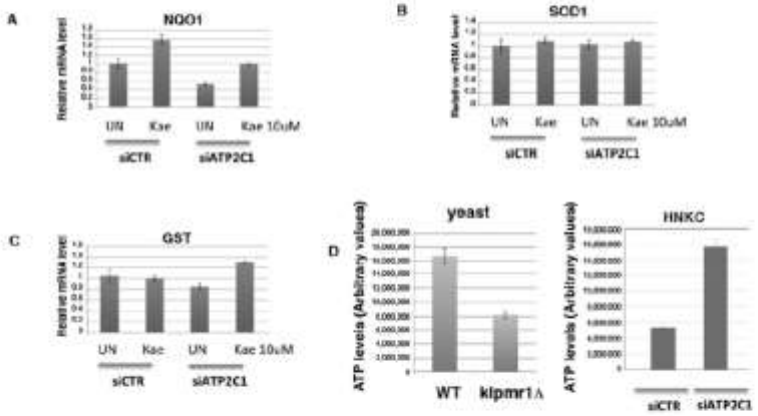

Figure 8. (**A**–**C**) NHKC cells were transfected with control (siRNA-CTR) or ATP2C1-specific siRNA oligonucleotides; 24 h later, cells were treated with Kaempferol (10 μM) for 24 h, the total RNA extracted, and the expression of the indicated targets analyzed by RT-PCR; (**D**) ATP production in both yeast (left) and primary human keratinocytes (right). ATP levels were analyzed in *Klpmr1Δ and WT* cells, and ATP production was assessed in primary human keratinocytes transfected with control (siRNA-CTR) or ATP2C1-specific siRNA oligonucleotides.

2.7. Increased Mitochondrial Activity as a Source of Oxidative Stress in ATP2C1-Defective Keratinocytes

Reactive oxygen species (ROS) are generated as a by-product of mitochondrial oxidative phosphorylation. However, inhibition of the mitochondrial electron transport induces generation of ROS that results in mitochondrial dysfunction [33]. NQO1 influences several aspects of mitochondrial function including: elevation of mitochondrial complex I activity; increased ATP production; maintenance of an elevated NAD/NADH ratio; and decreased ROS production ([33] and references therein). *ATP2C1* defective keratinocytes are characterized by reduced *NQO1* expression (Figure 8). Thus, we tested the ATP production in both *ATP2C1*-defective keratinocytes and *Klpmr1*Δ cells as a sign of mitochondrial function; we found that ATP production was decreased in *Klpmr1*Δ cells in line with the observed altered mitochondrial shape, indicating that mitochondrial dysfunction results in a cascade of events that include reduced ATP production (Figure 8D). Unexpectedly, we found that ATP production was increased in keratinocytes depleted of ATP2C1 function (Figure 8D). This observation indicates that in ATP2C1-defective mammals, cells with increased oxidative stress may be determined, at least in part, by increased mitochondrial activity, rather than by its dysfunction.

3. Discussion

Calcium [Ca^{2+}] serves as an ubiquitous second messenger in all eukaryotes [34]. There are several biological processes regulated by temporally and spatially defined changes of Ca^{2+} concentration in the cytoplasm or in defined organelles [34]. ATPase pumps modulate global cytosolic calcium levels and/or may control only the calcium levels, in particular intracellular calcium stores, e.g., endoplasmic reticulum, Golgi. In this context, one regulator of Golgi luminal calcium levels is the secretory pathway calcium ATPase 1 (SPCA1), an active transporter of calcium into the secretory pathway [4,35–38]. Mutations in *ATP2C1* (SPCA1) manifest as Hailey-Hailey disease, an autosomal dominant skin disorder [3]. Hailey-Hailey disease is characterized mainly by skin-specific phenotype symptoms characterized by the loss of cell-cell adhesion (acantholysis) [3]. Mutations in *ATP2C1* result in the decrease of ATP2C1 protein expression. There have been very few studies addressing the consequences of ATP2C1 inhibition in mammalian cells. In this context, yeast systems have become an attractive choice for the study of functionally conserved ATP2C1 function. We have developed a model yeast system to study the poorly defined genetic functions of the *ATP2C1* gene in Hailey-Hailey disease development. Cellular phenotypes associated with *ATP2C1/PMR1* loss of function in yeast can be investigated to clarify the cellular and molecular functions of *ATP2C1* in keratinocytes. In line with the notion that Ca^{2+} signal regulates a multitude of downstream responses, we show here that the strain bearing the *KlPMR1* gene disruption exhibited a pleiotropic phenotype. The pleiotropy of the mutant suggests that Pmr1 steers different calcium-dependent signal pathways to control distinct physiological processes. Here, we carried out a phenotypic screening to identify compounds able to revert either single or multiple phenotypes of *Klpmr1*Δ strain. Thus, understanding the mechanism of selective rescue by these compounds would shed light on the relevant molecular mechanisms to target for therapy. In this frame, we performed a pharmacological screening using a library of 131 natural molecules to analyze their ability to suppress the *Klpmr1*Δ phenotypes. Since the lack of the Golgi Ca^{2+}-ATPase in yeast, as well as in human HHD keratinocytes, induced a prominent increase of ROS production [20,23], we analyzed first the capability of the library to ameliorate the growth properties of the *Klpmr1*Δ strain under oxidative stress conditions. ROS conditions were achieved either exogenously by H_2O_2 administration or endogenously by menadione treatment. Six compounds, Indirubin (S2386), Cyclocytidine (S1973), Lappaconite Hydrobromide (S2387), Kaempferol (S2314), Nalidixic acid (S2328) and Azomycin (S2267) were able to reduce the sensitivity of *Klpmr1*Δ mutant to menadione and/or H_2O_2. The molecules S2328, S2386, S2314 and, more effectively, S2387 and S2267 decreased the growth defects of mutant cells in the presence of menadione. Meanwhile, S1973 and S2314 were more effective against H_2O_2. This indicates that the action of the different compounds depends on the localization of the ROS source. Then, we addressed if the six compounds selected in our screening were also able to rescue the multiple phenotypes associated with the deletion of the *PMR1* gene. Our findings indicated

that selected drugs were able to target the phenotypic traits of *Klpmr1*Δ mutant in both a multiple and phenotype-specific manner. Indeed, we found that Indirubin and Cyclocytidine, but not the other selected compounds, were able to revert the defect of *Klpmr1*Δ in regulation of intracellular calcium homeostasis as displayed by alterations in the sensitivity to Ca^{2+} chelator EGTA. Conversely, all six molecules were able to recover the defective wall structure of the mutant. However, we found that different molecules exhibited different strengths of suppression, with Nalidixic acid recovering cell wall morphology in 80% of *klpmr1*Δ cells. Similarly, we found that of the six selected compounds, three drugs (S2386, S2314 and S2387) restored the wild type-like tubular network of mitochondria. Among these compounds, only S2314 fully rescued the mitochondrial defects. The observation that compounds suppress different sets of phenotypes and at different strengths may suggest that Pmr1 loss of function elicits distinct and separable downstream responses. We have proven that yeast represents a useful model organism for investigating molecular and cellular aspects of Hailey-Hailey diseases, which may help to develop precise therapies for this disorder. Here we found six compounds able to ameliorate the multiple phenotypes of *klpmr1*Δ cells, summarized in the Supplementary Table S2.

Among the selected molecules, we found that Kaempferol was particularly effective for reverting the oxidative alterations of mutant cells, alleviating the sensitivity to ROS-generating compounds (H_2O_2 and menadione), decreasing the ROS cellular content, and restoring the mitochondrial function. Interestingly, we observed that Kaempferol treatment rescued the aberrant cell morphology and cell growth ability of siATP2C1-treated keratinocyte cells (Figures 5 and 6). Moreover, NRF2 expression was increased by Kaempferol treatment, further supporting our observation that loss of NRF2 activity in defective *ATP2C1* cells may have a direct effect on increased oxidative stress. Interestingly, treatment with Kaempferol reduced the oxidative stress of si*ATP2C1*-treated keratinocytes. Moreover, Kaempferol treatment induced an increase of NRF2 expression, as well as a reduction of oxidative stress, in si-ATP2C1-treated keratinocytes. These data further support our observation that loss of NRF2 activity in defective *ATP2C1* cells may have a direct effect on increased oxidative stress. ROS are generated as a by-product of mitochondrial oxidative phosphorylation. Here we found that mitochondrial integrity is altered by inactivation of the *K. lactis PMR1* gene, and Kaempferol treatment led to the rescue of mitochondrial phenotype together with decreased sensitivity to ROS sources. Thus, we tested the mitochondrial functionality of both *klpmr1*Δ cells and siATP2C1-interfered human keratinocytes by analyzing ATP production. In line with the altered mitochondrial morphology, ATP production was decreased in *Klpmr1*Δ cells (Figure 8D). Unexpectedly, we found that ATP production was increased in keratinocytes depleted of ATP2C1. Stimulation of mitochondrial oxidative metabolism by Ca^{2+} is now generally recognized as an important mechanism for the control of cellular ATP homeostasis. Increases in cytosolic calcium results in an increased mitochondrial Ca^{2+} uptake and ATP synthesis [33]. Thus, it may be possible that in ATP2C1-defective mammals, the increased oxidative stress of cells is determined by increased mitochondrial activity, rather than by its dysfunction.

4. Materials and Methods

4.1. Yeast Strains, Growth Conditions

The strains used in this study were MW278-20C (MAT a, ade2, leu2, uraA) and CPK1 (MAT a, ade2, leu2, uraA, PMR1::KanR). The yeast growth media used for all the experiments was YPD medium (1% yeast extract, 1% peptone, 2% glucose, DIFCO (Difco, Becton Dickinson, Sparks, MD, USA)).

4.2. Library Screen

For the screening, a library of 131 natural products was purchased from Selleck Chemicals (Houston, TX, USA). The complete list of chemicals screened is provided in Supplementary Table S1. Compounds were stored as 10 mM stock solutions in dimethyl sulfoxide (DMSO) at −20 °C until use. Compound stocks were diluted in a volume of 1 mL of YPD to the indicated concentrations. After 24 h of growth at 28 °C, five-fold serial dilution of cultures were spotted onto YPD agar plates

supplemented or not with 60 µM menadione or 4 M H_2O_2 or 20 mM EGTA, as indicated. The plates were incubated at 30 °C for 3 days. To determine a dose–response curve, mutant cells were treated at 30 °C for 24 h with increasing concentrations of each chemical diluted in 1 mL of YPD at the indicated concentrations. After that, cells were diluted to 0.07 OD_{600} in 2 mL of YPD containing 3 mM H_2O_2 or 20 µM menadione. After 24 h of growth at 30 °C the optical density (OD) was measured. The EC50 of the compounds was obtained from the dose–response curves using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

4.3. Fluorescence Microscopy

After 24 h of treatment with the molecules, the yeast cells grown in YPD medium were harvested, washed with water and then mixed 1:1 with the vital dye 2-(4-dimethylaminostyryl)-*N*-methylpyridinium iodide (DASPMI) as described in [39]. The chitin staining was performed using the probe Calcofluor White (CFW) by the method of [17]. Epifluorescence microscopy was carried out with a Zeiss AxioVert 25 microscope fitted with a ×100 immersion objective and a standard filter set.

4.4. Primary Human Keratinocytes

Primary human keratinocytes were purchased from (Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained in modified low calcium medium (EpiLife, Thermo Fisher, Waltham, MA, USA). Cells at passages 1 and 2 were used for study purposes.

4.5. Cell Culture and Transfection

Primary human keratinocytes and HaCaT cells (70–80% confluent) were maintained in modified low-calcium medium and transfected using the Lipofectamine -RNAiMAX transfection Reagent according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Primary keratinocytes were transfected with 100 nmol·L^{-1} small interfering RNAs (siRNAs) for validated human ATP2C1 (L-006119-00; Thermo Scientific/Dharmacon, Lafayette, CO, USA) and the corresponding control scrambled siRNAs cells were analyzed at the indicated times after transfection by either CMH2DCFDA analysis for ROS detection or Western blot as indicated [23].

4.6. Reagents and Immunoblotting

ATP2C1 antibodies were purchased from Abcam (Cambridge, MA, USA) and NOTCH1 (N1Val) and NRF2 were purchased from Cell Signaling Technology (Beverly, MA, USA). All cell extracts were prepared according to the manufacturer's instructions for detection of phosphor-ERK (Cell Signaling Technology, Beverly, MA, USA) as previously described [30]. Adenosine triphosphate (ATP) content of keratinocytes was determined using the luciferin reaction, ATP Determination Kit (Thermo Fisher Scientific, Waltham, MA, USA). A standard curve was made by using solutions containing increasing concentrations of ATP.

4.7. RNA Analysis and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from cells in guanidine isothiocyanate (Trizol reagent, Thermo Fisher Scientific, Waltham, MA, USA) and further processed by reverse transcriptase polymerase chain reaction (RT-PCR) as described in [40]. Each sample was analyzed in triplicate by qRT-PCR and in at least three independent experiments. qRT-PCR was performed at the opportune annealing temperature with the primers indicated in Table 2 with SensiFAST SyBr Hi-ROX kit (Bioline, London, UK) or with specific TaqMan MGB primers/probe using Taqman gene expression assay (Thermo Fisher Scientific, Waltham, MA, USA)

Table 2. qRT-PCR primers.

SyBr Green Assays	Sequence 5′-3′
GST-M1 Fw	AGAGGAGAAGATTCGTGTGG
GST-M1 Rev	TGTTTCCTGCAAACCATGGC
GAPDH Fw	TGCACCACCAACTGCTTAG
GAPDH Rev	GAGGCAGGGATGATGTTC
Taqman Gene Expression Assays	**Assay Reference Number**
NFE2L2 (NRF2)	Hs00975961_g1
GAPDH	Hs99999905_m1
NQO1	Hs02512143_s1
SOD1	Hs00533490_m1

5. Conclusions

In this work we have explored the use of the yeast *K. lactis* as a tool to identify specific compounds that target specific cellular phenotypes and obtain more insight into mechanisms of disease pathology by probing the mechanisms involved in their action. Oxidative stress represents a hallmark of both *Kluyveromyces lactis* lacking *PMR1* and keratinocytes derived from the lesional areas of HHD patients and it could be associated with the decreased action of the transcription factor NRF2, involved in the regulation of several detoxifying factors. Our results indicated that molecules able to promote activation of this pathway might provide protection to the HHD-skin cells.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/19/6/1814/s1.

Author Contributions: G.F., A.Z., S.C. performed the experiments and collected the data. C.T. and D.U. conceived, designed and supervised the study. G.F., A.Z., S.C., C.T., D.U. analyzed the data. G.F., A.Z., S.C., C.T., D.U. assembled the figures. G.F. wrote the first draft of the manuscript. C.T. and D.U. wrote the manuscript. All the authors contributed in manuscript writing and editing and approved the final version of the manuscript.

Acknowledgments: This work was supported by the Fondazione Telethon GGP12264 and by the Italian Association of Hailey-Hailey disease, AMaHHD Onlus.

Conflicts of Interest: The authors declare no conflicts of interest.

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Hypotonic, Acidic Oxidizing Solution Containing Hypochlorous Acid (HClO) as a Potential Treatment of Hailey-Hailey Disease

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Academic Editor: Valeria Amendola
Received: 1 October 2019; Accepted: 2 December 2019; Published: 4 December 2019

Abstract: Hailey–Hailey disease (HHD) is a rare, chronic and recurrent blistering disorder, characterized by erosions occurring primarily in intertriginous regions and histologically by suprabasal acantholysis. Mutation of the Golgi Ca^{2+}-ATPase *ATP2C1* has been identified as having a causative role in Hailey–Hailey disease. HHD-derived keratinocytes have increased oxidative-stress that is associated with impaired proliferation and differentiation. Additionally, HHD is characterized by skin lesions that do not heal and by recurrent skin infections, indicating that HHD keratinocytes might not respond well to challenges such as wounding or infection. Hypochlorous acid has been demonstrated in vitro and in vivo to possess properties that rescue both oxidative stress and altered wound repair process. Thus, we investigated the potential effects of a stabilized form of hypochlorous acid (APR-TD012) in an in vitro model of HHD. We found that treatment of ATP2C1-defective keratinocytes with APR-TD012 contributed to upregulation of Nrf2 (nuclear factor (erythroid-derived 2)-like 2). Additionally, APR TD012-treatment restored the defective proliferative capability of siATP2C1-treated keratinocytes. We also found that the APR-TD012 treatment might support wound healing process, due to its ability to modulate the expression of wound healing associated cytokines. These observations suggested that the APR-TD012 might be a potential therapeutic agent for HHD-lesions.

Keywords: Hailey–Hailey disease; oxidative-stress; keratinocytes

1. Introduction

Hailey–Hailey disease (HHD, OMIM 16960), also indicated as benign familial pemphigus, is an autosomal dominantly inherited dermatosis manifesting in the 3rd to 4th decades of life [1]. The overall incidence and prevalence of HHD is unknown although some authors have reported an incidence between 1:40,000 and 1:50,000 [2,3]. HHD is a characterized by red scaly areas that can be painful and itchy and can lead to superficial blisters and eroded areas of the skin. This disease often has a remission and recurrence pattern, which may be constant in some patients. HHD is associated with the loss of a single copy of *ATP2C1*, a gene that is likely essential in humans, as more severe phenotypes are found in patients who suffer clonal loss of both copies of the gene. Consistently, mice embryos homozygous for null mutations in ATP2C1 die with defects in neural tube closure, while heterozygotes show susceptibility to squamous cell tumors, a phenotype observed rarely in humans with Hailey–Hailey [4,5]. The gene, located on the long arm of chromosome 3, 3q21-q24

region, encodes the human secretory pathway Ca^{2+}/Mn^{2+} ATPase, hSPCA1 [6]. Although ATP2C1 is mostly localized to the Golgi apparatus, it regulates also endoplasmic reticulum (ER) Ca^{2+} stores with effects on both Golgi and ER functions. In keratinocytes the lack of ATP2C1 leads to the loss of cell-to-cell adhesion (acantholysis) among the cells of the suprabasal layer of epidermis probably due to a retraction of keratin intermediate filaments from the desmosomal plaques [1]. Although ATP2C1 mutations are 100% penetrant, currently there is no treatment known to be effective in reducing the cutaneous manifestations of HHD. The standard of care (SOC) treatment consists in either topical or oral administration of corticosteroids often used in combination with topical/systemic antimicrobial agents. External factors, such as sweating, UV exposure, friction and superinfection with bacteria, fungi and viruses play an important role in exacerbations and persistence of lesions [2,7,8]. Calcium regulates the proliferation and differentiation of keratinocytes both in vivo and in vitro, thus it is not surprising that ATP2C1 mutations affect the skin. However, it is unclear how ATP2C1 loss selectively affects keratinocyte homeostasis. Oxidative stress represents a hallmark of the keratinocytes derived from the lesions of HHD patients and it could be associated to the decreased action of some detoxifying systems [9–13]. Particularly, in the lesional-derived keratinocytes of HHD patients, the expression of detoxifying genes is down-regulated [9–13]. As oxidative stress is thought to play a pivotal role in promoting the skin lesions of Hailey–Hailey, counteracting oxidative-stress could be a viable therapeutic approach for HHD. HHD lesional-derived keratinocytes in addition to being characterized by increased oxidative stress also show decreased expression levels of both NOTCH1 and NRF2 [9–13]. However, in vitro, the inhibition of ATP2C1 expression in HaCaT cells resulted in increased levels of activated NOTCH1 and decreased expression of NRF2, indicating that NOTCH1 downregulation in HHD-lesions could represent a secondary event that might be required at a later stage of the lesion development [10]. Both NOTCH1 and NRF2 factors are important determinant of skin homeostasis; NOTCH signaling is an essential regulator of keratinocyte growth and differentiation and NRF2 activation regulates antioxidant genes transcription directly. Skin lesions that do not heal and by recurrent skin infections are main pathological feature of HHD keratinocytes, indicating that they may be not able to counteract insults such as wounding or infection. After injury an increase of local cytokine production from keratinocytes occurs [14–16]. In skin wounds in order to regulate the re-epithelization process, crucial cytokines as interleukin (IL)-6 and transforming growth factor (TGF)-beta, are produced locally [17–23]. It has been shown that HHD lesion-derived keratinocytes were defective in wound-induced cytokine production [10]. Therefore, these data indicate that keratinocytes derived from the HHD lesions are defective in managing both oxidative-stress response and wound signal that ultimately could contribute to the poor healing of HHD lesions. NRF2 is widely regarded to be an oxidative stress-activated transcription factor and its essential role is to keep a physiological redox homeostasis. Transient and moderate oxidative stress may up-regulate genes involved in antioxidant and cytoprotective pathways through the activation of the transcription factor Nrf2 [24]. Hypochlorous acid (HClO) in addition to a strong antimicrobial activity is an oxidant generated in the pathogenesis of many disorders [25,26]. After exposure of cells to HClO, NRF2-mediated antioxidant response is activated and resulted in increased protein levels of NRF2, as well as in an increase in the expression of its target genes [25–27]. In addition HClO has both pro-inflammatory and anti-inflammatory properties [25–27]. APR TD012 is a water-based solution obtained by an electrolysis process (Tehclo Technology). This method allows us to obtain the active compound HClO deliverable in a hypotonic, acidic (pH: 2.5–3.0) and oxidizing (Oxidation Reduction Potential: 1000–1300 mV) solution. Due to the great relevance of both oxidative stress and altered pattern of cytokines expression in HHD-derived keratinocytes, we evaluated if the APR TD012 treatment was able to recover the oxidative-stress as well as the altered pattern of cytokine expression of ATP2C1-defective keratinocytes. With this aim, we analyzed the response of ATP2C1-defective keratinocytes to the addition of this hypotonic acidic oxidizing solution containing HClO. We investigated the effect of APR TD012 treatment on proliferation rate, NRF2 expression, antioxidative-stress activity and cytokines expression of ATP2C1-defective keratinocytes. We found that APR TD012 solution was able to restore NRF2 defective expression, differentially affected the

expression of TGFbeta1 and TGFbeta2 and had a favorable effect on ATP2C1-defective keratinocyte proliferation and on in vitro wound assay. These features, together with the well-known antimicrobial activity of HClO, identify APR TD012 as a potential agent in the treatment of HHD-lesions.

2. Results

2.1. Levels of Oxidative Stress in ATP2C1 Defective Keratinocytes Treated with APR TD012

To test the effect of hypotonic acidic oxidizing solution containing HClO (APR TD012) we used small interfering RNA (siRNA)-ATP2C1-treated keratinocytes as an in vitro model of Hailey–Hailey disease. Towards this aim, HaCaT cells were transfected with siCTR and siATP2C1 and then treated with APR TD012. As previously observed, we found that ATP2C1 loss increased oxidative stress (Figure 1A,B). After 24 h of transfection, the percentage of 2′, 7′-dichlorofluorescin diacetate (DFCA)-positive cells in siATP2C1 cells reached ≅ 38%, whereas only ≅ 15% of the siRNA-CTR control cells were DFCA-positive. In this model analysis of Reactive Oxygen Species (ROS) levels, APR TD012 treatment brought an increased oxidative-stress in both siCTR (≅28%) and siATP2C1 (≅52%) treated HaCaT cells (Figure 1B). At the 48 h time point of transfection, the percentage of DFCA-positive cells in both siCTR and siATP2C1 cells were similar to ROS levels observed at 24 h (Figure 1B), whereas the levels declined in both siCTR and siATP2C1 cells when APR TD012 was present in the medium (Figure 1B). Interestingly, we reported that HHD lesion-derived keratinocytes were hypo-proliferative compared to the non-lesion-derived keratinocytes [10]. HClO and hypotonic stress has been shown to have a positive effect on keratinocyte migration and proliferation [25], and both these events are defective in Hailey–Hailey disease [10]. Therefore, we tested whether APR TD012 treatment could influence ATP2C1-defective keratinocytes proliferation. We confirmed that siATP2C1 cells had reduced proliferation compared to siCTR treated cells (Figure 1C). Interestingly the treatment of siATP2C1 cells with APR TD012 rescued the defective proliferation of siATP2C1-treated HaCaT cells (Figure 1C).

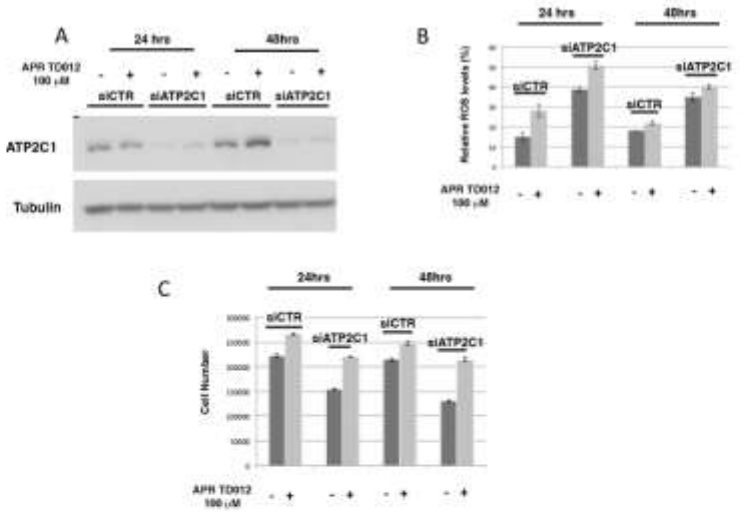

Figure 1. ROS and proliferative rate after APR TD012 treatment. (**A**) Immunoblot analysis of ATP2C1, in siCTR and siATP2C1 HaCaT cells. Tubulin expression was used as control for equal loading. (**B**) Cells were analyzed by Fluorescence Activated cell sorting (FACS) and % of ROS levels is shown. (**C**) Cell number of siCTR and siATP2C1 keratinocytes was analyzed by Trypan-blue assay. The averages ± standard error of two independent experiments in triplicate are shown.

2.2. Effects of APR TD012 on the NRF2/Antioxidant Defense Pathway

Previously we have observed that NOTCH1 expression levels were increased in siATP2C1 cells [10]. Additionally, the expression of NRF2 was decreased in siATP2C1 treated keratinocytes [10].

These events could play an important role in HHD development since ATP2C1 loss would trigger a mechanism that results in NOTCH1 activation and DNA damage response inhibition. As a result of NFR2 down-modulation increased ROS levels produced DNA damage up to a threshold that keratinocytes could not repair, which would then promote lesion manifestation. Thus, we investigated how a treatment with hypotonic acidic oxidizing solution containing HClO might affect both NOTCH1 and NRF2 expression. We found that siATP2C1 treatment of HaCaT keratinocytes showed an increased expression of active NOTCH1 when compared to control cells (Figure 2A). However, there were no significant differences between vehicle and APR TD012 treated siATP2C1-cells. Conversely, the NRF2 protein expression levels were significantly higher after treatment with APR TD012 (Figure 2B). In conjunction with NRF2 increased expression, we observed that APR TD012 treatment induced the expression of NRF2 target gene NQO1 in both HaCaT and primary human keratinocytes (Figure 2C,D). These data suggest that APR TD012 promoted an antioxidant defense response by activating the NRF2 pathway although this protective circuitry failed to counteract the high ROS levels observed in ATP2C1-defective keratinocytes.

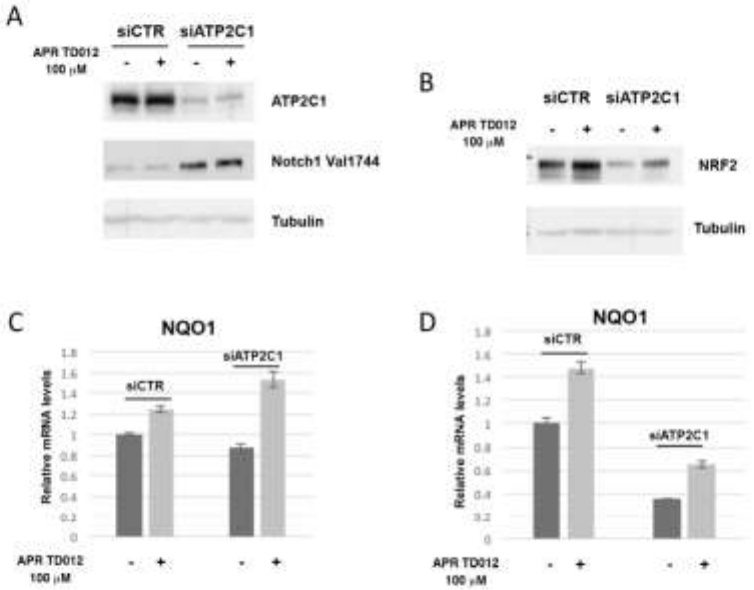

Figure 2. Effects of APR TD012 on NRF2 and its target expressions. Immunoblot analysis of ATP2C1, Notch1 Val1744 (**A**) and NRF2 (**B**) in siCTR and siATP2C1 HaCaT cells. Tubulin expression was used as control for equal loading. qRT-PCR analysis of NQO1 mRNA expression levels in siCTR and siATP2C1 HaCaT (**C**) or NHEK (**D**) cells. The values are expressed as fold changes of siATP2C1 cells vs. the siCTR. The averages ± standard error of two independent experiments are shown.

2.3. *Effects of APR TD012 on the Expression of Keratinocyte-Derived Cytokines*

The success of the wound healing process depends on growth factors, cytokines and chemokines. HHD lesions are characterized by deregulated cytokine expression and decreased repair properties [10]. Thus, we investigated the influence of APR TD012 treatment on the pattern of deregulated cytokines on ATP2C1-defective keratinocytes. We have shown an altered expression levels of the pro-inflammatory cytokines IL-1, IL-6, IL-8, TGFβ1 and TGFβ2 in ATP2C1 defective keratinocytes [10]. However, no significant differences in IL-1, IL-6 and IL-8 levels were observed between vehicle and APR TD012 treated siATP2C1-cells (Figure 3A–C); Conversely, in the siATP2C1 cells the mRNA levels of TGFβ1 and TGFβ2 were significantly higher than those of the control siCTR-control cells (Figure 3D,E). Interestingly, a significant difference in TGFβ1 and TGFβ2 levels were observed between the vehicle and APR TD012 treated cells. In particular, the upregulation of TGFβ1 expression was observed in the

siATP2C1 cells compared to the siCTR cells and, interestingly, even more after APR TD012 treatment (Figure 3D).

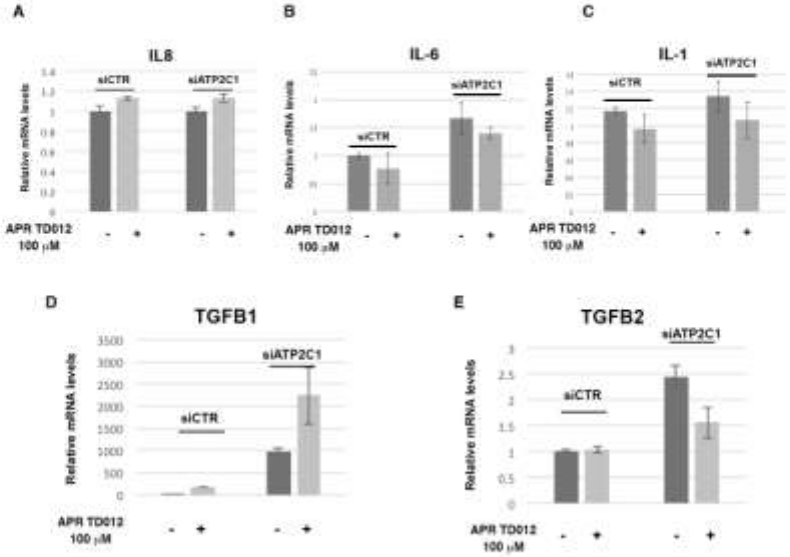

Figure 3. Effects of APR TD012 solution on proinflammatory cytokine in ATP2C1 defective keratinocytes. (**A**) IL-8, (**B**) IL-6, (**C**) IL-1 (**D**) TGFβ-1 and (**E**) TGFβ2 were quantified by q-RT-PCR assay. The values are expressed as fold changes of siATP2C1 cells vs. the siCTR. Data are expressed as mean ± SD of two independent experiments performed in triplicate.

Likewise, the levels of the TGFβ2 cytokine were significantly higher in the siATP2C1 treated cells than those of the control cells (Figure 3E). On the contrary, while the TGFβ2 expression remained unaffected in siCTR cells, a decreased expression levels were observed between the vehicle treated and APR TD012 treated siATP2C1-cells (Figure 3E). These data suggest that APR TD012 might influence the pattern of proinflammatory cytokines expression in HHD-keratinocytes.

2.4. In Vitro Wound Healing Potential of APR TD012 on ATP2C1-Defective Keratinocytes

HHD keratinocytes are characterized by impaired healing repair [10] and migration of cells is critically involved in wound repair; thus we conducted a scratch assay to analyze the healing process of siATP2C1-treated keratinocytes in the presence of APR TD012. After 24 h exposure to the APR TD012 solution, we observed that in the control siCTR-treated cells, the rate of migration was not influenced by the treatment (Figure 4). The values given were calculated based on the scratch coverage after 24 h. The migration analysis showed that for the siATP2C1 cells the vehicle-treated cells showed a significant decrease in migration with the value of 50%, when compared with the siCTR-treated cells. When the APR TD1012 treated cells were compared, they showed a significant increase in migration with the migration rate of 34% when compared to siATP2C1 vehicle-treated cells, also if there was still difference when compared with siCTR-control cells.

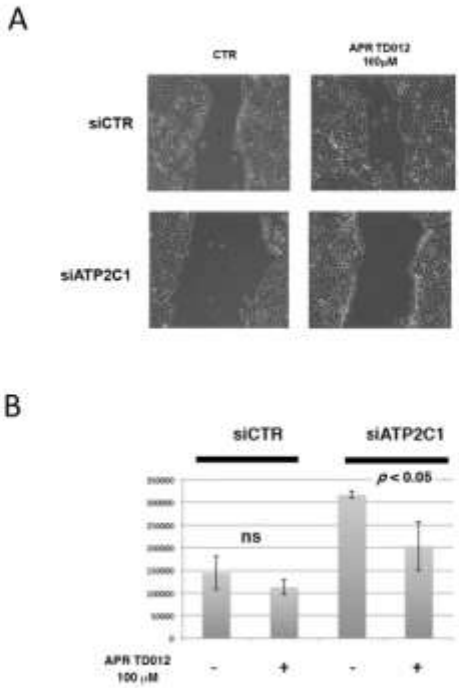

Figure 4. In vitro scratch assay (×40 magnification). (**A**) Representative images of siCTR and siATP2C1 cell migration into the scratched area after treatment with vehicle or APR TD012. (**B**) Quantitative analysis of the migration rate was analyzed with the use of ImageJ software. Data are expressed as mean ± standard deviation from three individual experiments.

The results showed that the proliferation and migration of siATP2C1 treated keratinocytes were significantly higher in APRTD012 cells than the control group. These data suggested that APR TD012 enhanced ATP2C1 defective keratinocyte proliferation and migration.

3. Discussion

HClO is thought to promote wound-healing process and to both promote oxidative-stress and consequently the activation of the NRF2/antioxidant pathway [15,25–27]. We have found that both these events are defective in Hailey–Hailey disease [10]. Our previously published results had implicated oxidative stress and the response to it as contributing factors to the presentation of HHD. It is known that transient and moderate oxidative stress may up-regulate genes involved in antioxidant and cytoprotective pathway through the activation of the transcription factor Nrf2 [24]. We hypothesized that APR TD012, a hypotonic acidic oxidizing solution containing HClO might be an effective treatment for HHD by restoring the activation of the NRF2 pathway. Additionally, previous observations suggested that HClO improves wound healing process since is highly active against bacterial, viral and fungal human pathogens [15,25–27]. HHD is characterized by skin lesions that do not heal and by recurrent skin infections, indicating that HHD keratinocytes might not respond well to challenges such as wounding or infection. Thus in this study, we aimed to assess the potential use of APR TD012 in patients with HHD. To test this hypothesis, we first analyzed whether there was evidence of an effect of hypotonic acidic oxidizing solution containing HClO treatment in an in vitro model of HHD. We first investigated the effects of APR TD012 treatment on the oxidative-stress levels of ATP2C1 defective-keratinocytes. We found that oxidative-stress increased in APR TD012 treated keratinocytes with both functional and defective ATP2C1. It has been reported that the oxidative action of HClO induces the activation of the NRF2/antioxidant defense pathway thereby, paradoxically, reducing oxidative-stress [15,25–27]. Thus, to determine a possible functional link between APR TD012

and NRF2 and the presence of oxidative stress in HHD, we performed a series of experiments in HaCaT keratinocytes transfected with either siCTR or siATP2C1 and then treated with either vehicle or APR TD012. As previously shown, we found that NRF2 was downregulated in keratinocytes with interfered *ATP2C1* gene. Interestingly, when siATP2C1 transfected keratinocytes were treated with APR TD012, NRF2 expression was restored. We found that after treatment, the proliferation of ATP2C1 defective keratinocytes resembled that of control keratinocytes. Together, these results indicate that APR TD012 solution can act directly on keratinocytes to protect them from HHD defects, consistent with previous observation suggesting that increased NRF2-pathway increases the defense mechanism of HHD-keratinocytes [9,10].

An important finding of our study was the observation that APR TD012 influenced the expression of both TGF-β isoforms β1 and β2. Although TGF-β isoforms signal through the same cell surface receptors, they display distinct functions during wound healing in vivo through mechanisms that have not been fully elucidated [28,29]. Numerous studies have highlighted the role of TGF-beta signal in cutaneous wound healing [28,29]. The well-characterized role of TGF-β1 and -β2 on promoting wound healing has provided the basis for the use of TGF-β1 or -β2 as potential therapeutic [28,29]. Interestingly, we found that in ATP2C1 defective cells, APR TD012 treatment decreased TGF-β2 expression while increased TGF-β1 expression. Although it is likely more complex than this, since TGF-β isoforms display distinct functions during wound healing, it is thought that the ratio of TGF-β isoforms will differently influence the wound healing process [30]. Thus, also if we did not address this aspect our results indicate that APR TD012 treatment altering the ratio of TGF-β isoforms could positively affect the resolution of HHD lesions. It has been found that loss of TGFβ-2 signaling in keratinocytes led to an accelerated re-epithelialization of full thickness excisional wounds accompanied by an increased proliferation in keratinocytes at the wound edge [31]. Furthermore, impaired TGFβ signaling in keratinocytes reduces apoptosis in re-epithelialized wounds of transgenic animals [31]. A speculative prospective is represented by the possibility that the ability of APR TD012 to decrease TGF-β2 while increasing TGF-β1 expression could be a means to restore the proliferative potential of ATP2C1 defective keratinocytes improving the wound process. To support this hypothesis, we performed a scratch wound healing assay after APR TD012 treatment. The results showed a reduction of width of wound in siATP2C1 cells demonstrating an improved proliferation through this pharmacological approach. The wound healing requires elimination of microrganisms, removing damaged cells and tissue and restoring the skin barrier, three needed steps to restore tissue integrity and APR TD012 might help to carry out these complex of processes.

Together, these results provided a rationale to test the use of APR-TD012 solution for the treatment of HHD lesions.

4. Materials and Methods

4.1. Cell Culture

HaCaT keratinocyte-derived cell line were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 5% L-Glutamine, 2% penicillin and streptomycin, at 37 °C with 5% CO^2.

4.2. Cell Culture and Transfection

HaCaT cells (70–80% confluent) were transfected using the Lipofectamine RNAiMAX transfection Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) using 100 nmol L^{-1} small interfering RNAs (siRNAs) for validated human ATP2C1 (L-006119-00; Thermo Scientific/Dharmacon, Lafayette, CO, USA) and corresponding control scrambled siRNAs. Cells were analyzed after 48 h of transfection for ROS detection or Western blot as indicated. In the time 24 and 48 h point experiment HaCaT cells (20–30% confluent) were incubated 6 h with the Lipofectamine RNAiMAX transfection reagent according to manufacturer's instructions (Thermo

Fisher Scientific, MA USA). Then cells were untreated or treated with APR TD012 solution for either 24 or 48 h and analyzed for ROS detection or Western blot as indicated.

4.3. Cell Treatment with APR TD012

APR TD012 (batch 2147) was diluted 1:10 in order to reach the concentration of 100 μM in the assays.

4.4. Cell Viability Assay

HaCaT cells (siCTR and siATP2C1) were grown and used for cell viabilities assay at the second passage by using Trypan-blue based assay. After 24 h of transfection with siATP2C1 or siCTR, cells were treated 24 h with 100 μM APR TD012. As control samples, cells were treated with equal volumes of the vehicle (H2O). Trypan-blue assays were performed in technical triplicates and figures show the averages ± SEM of at least two biological replicates.

4.5. Measurement of ROS Accumulation

Intracellular production of ROS was measured using cell-permeable fluorescent dyes, 5-(and-6)-chloromethyl-2′, 7′dichlorodihydrofluorescein diacetate, acetyl ester (CMH2DCFDA Molecular Probes). When this dye is oxidized by ROS in cells, their fluorescent signals increase. For the assay, after transfection and addiction of APR TD012, HaCaT cells were treated with CMH2DCFDA for 30 min, in the dark at 37 °C. Next, cells were washed twice with PBS, trypsinized and fluorescence was measured using flow cytometry (excitation at 488 nm, emission at 515–545 nm). Data analysis was performed with CellQuestPro software (BD Biosciences, Milan, Italy), and the mean fluorescence intensity was used to quantify the responses. A minimum of 10,000 cells were acquired for each sample, excluding the dead population.

4.6. Western Blot Assay

Cells were lysed in Tris HCl 20 mM pH 7.5, NaCl 150 mM, EDTA 1 mM pH 8, Triton 1%, NaF 30 mM, Na3VO4 1 mM, PMFS 1mM and protease inhibitors (Merck life Science, Milan Italy); samples were centrifuged at 13,000 rpm for 15 min and supernatant was collected. Quantification was performed with Bradford assay (Bio-Rad). Lysates were denatured at 95 °C and separated through SDS-PAGE on 8% acrylamide gel. After transfer to a polyvinylidene difluoride (PVDF) membrane, proteins were immunoblotted using standard procedures. The primary antibodies for ATP2C1 and NRF2 were purchased from [10] Abcam, Cambridge, UK; Notch1Val1744and Tubulin were purchased from Cell Signaling Technology, Beverly, MA, USA and Sigma Aldrich, Milan, Italy respectively.

4.7. RNA Analysis and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from cells, in guanidine isothiocyanate (Trizol reagent, Thermo Fisher Scientific, MA USA) and further processed by reverse transcriptase polymerase chain reaction (RT-PCR) as described [32]. Each sample was analyzed in triplicated by qRT-PCR and in at least two independent experiments. qRT-PCR was performed at the opportune annealing temperature with the primers indicated below, with SensiFAST SyBr Hi-ROX kit (Bioline, UK) or with specific TaqMan MGB primers/probe using Taqman gene expression assay (Thermo Fisher Scientific, MA USA). hIL-1 and hIL-6 primers were previously described in [10].

4.8. Statistical Analysis

Each experiment was repeated at least two times independently. All results were expressed as means SD, and $p < 0.05$ was used for significance. One-Way ANOVA analysis for independent samples was used to determine statistical significance.

4.9. Primers

hTGFB1 qPCR Fw:	CAGAAATACAGCAACAATTCC;
hTGFB1 qPCR Rev:	CTGAAGCAATAGTTGGTGTC;
hIL8 qPCR Fw:	AAGGAAAACTGGGTGCAGAG;
hIL8 qPCR Rev:	ATTGCATCTGGCAACCCTAC;
hGAPDH qPCR Fw:	TGCACCACCAACTGCTTAG;
hGAPDH qPCR Rev:	GAGGCAGGGATGATGTTC;
TGFbeta2: Hs00234244_m1.	

5. Conclusions

Hailey-Hailey disease (HHD) is a Rare disease and currently there is no treatment known to be effective in reducing the cutaneous manifestations of the disease. We have gathered compelling evidence indicating that oxidative-stress plays a pivotal role in promoting the skin lesions of Hailey–Hailey disease. HHD-keratinocytes show decreased expression levels of NRF2 and NRF2-regulated antioxidant enzymes leading to the accumulation of ROS. The standard of care (SOC) treatment consists in either topical or oral administration of corticosteroids often used in combination with topical/systemic antimicrobial agents, but without reversing the pathological process. In this line, the discovery of new therapies aimed to target the pathogenic mechanism underlying the disease is undoubtedly an important goal, in order to provide better and more efficient treatment conditions for HHD patients. In this context, we investigated the potential effects of a stabilized form of hypochlorous acid (APR-TD012) in an in vitro model of HHD. We found that treatment of ATP2C1-defective keratinocytes with APR-TD012 contributed to up-regulation of Nrf2. Additionally, APR TD012-treatment restored multiple defects observed in siATP2C1-treated keratinocytes. These observations suggested that the APR-TD012 might be a potential therapeutic agent for HHD-lesions.

Author Contributions: Conceptualization, S.C. (Samantha Cialfi) and C.T.; Investigation, S.C. (Samantha Cialfi), S.C. (Salvatore Calabro), M.F., A.Z.; resources, I.S.; data curation, S.C. (Samantha Cialfi) and C.T; writing—Original draft preparation, S.C. (Samantha Cialfi) and C.T.; writing—Review and editing, C.T. funding acquisition, C.T.

Funding: This work was supported by the Fondazione Telethon GGP12264 and by the Italian Association of Hailey-Hailey disease, AMaHHD Onlus.

Acknowledgments: APR-TD012 solution was kindly provided by APR Applied Pharma Research Balerna, Switzerland.

Conflicts of Interest: APR-TD012 solution was kindly provided by APR Applied Pharma Research Balerna, Switzerland. The company had no role in the design, execution, interpretation, or writing of the study.

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Sample Availability: Samples of all compounds are available from the authors.

JBC ARTICLE

✖ Author's Choice

PLK1 targets NOTCH1 during DNA damage and mitotic progression

Received for publication, June 21, 2019, and in revised form, September 25, 2019 Published, Papers in Press, October 9, 2019, DOI 10.1074/jbc.RA119.009881

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Edited by Patrick Sung

Notch signaling plays a complex role in carcinogenesis, and its signaling pathway has both tumor suppressor and oncogenic components. To identify regulators that might control this dual activity of NOTCH1, we screened a chemical library targeting kinases and identified Polo-like kinase 1 (PLK1) as one of the kinases involved in arsenite-induced NOTCH1 down-modulation. As PLK1 activity drives mitotic entry but also is inhibited after DNA damage, we investigated the PLK1-NOTCH1 interplay in the G_2 phase of the cell cycle and in response to DNA damage. Here, we found that PLK1 regulates NOTCH1 expression at G_2/M transition. However, when cells in G_2 phase are challenged with DNA damage, PLK1 is inhibited to prevent entry into mitosis. Interestingly, we found that the interaction between NOTCH1 and PLK1 is functionally important during the DNA damage response, as we found that whereas PLK1 activity is inhibited, NOTCH1 expression is maintained during DNA damage response. During genotoxic stress, cellular transformation requires that promitotic activity must override DNA damage checkpoint signaling to drive proliferation. Interestingly, we found that arsenite-induced genotoxic stress causes a PLK1-dependent signaling response that antagonizes the involvement of NOTCH1 in the DNA damage checkpoint. Taken together, our data provide evidence that Notch signaling is altered but not abolished in SCC cells. Thus, it is also important to recognize that Notch plasticity might be modulated and could represent a key determinant to switch on/off either the oncogenic or tumor suppressor function of Notch signaling in a single type of tumor.

NOTCH signaling is essential for development, and it is a type of cell-cell signaling that participates in a wide range of biological processes from neurodegeneration to tumorigenesis (1, 2). The canonical *NOTCH* pathway is mediated by the regulated intramembrane proteolysis pathway, in which NOTCH receptors undergo ligand-dependent sequential endoproteolysis via different enzymes, including presenilin (PS)[4]/γ-secretase (3). The NOTCH-1 intracellular domain (ICD), which is produced by PS/γ-secretase–mediated cleavage at site 3 within the transmembrane domain, translocates to the nucleus to activate transcription of target genes (1, 2). Alteration of *NOTCH* signaling has been described as a major player in several human cancers (4). Furthermore, multiple lines of evidence indicate that *NOTCH* signaling is not exclusively oncogenic but can act as a tumor suppressor. In animal models, evidence for *NOTCH* signaling in mediating each of these roles has been established. Additionally, the NOTCH1 tumor suppressor role is also underlined by the loss or inactivating mutations of members of the *NOTCH* signaling pathway in human cancers, particularly in head and neck squamous cell carcinoma (HNSCC), in which inactivating mutations of *NOTCH1* were found in 10–15% of the tumors (5–10). Interestingly, a subset of HNSCC tumors with the *NOTCH1* WT sequence exhibit a *NOTCH* pathway copy number increase with activation of the downstream NOTCH targets, *HES1*/*HEY1* (5, 10). Additionally, inhibition of *NOTCH1* or *HEY1* significantly decreased cell growth of primary tumor-derived cells, indicating their potential involvement in HNSCC development (5, 10, 11). The molecular regulation of the dichotomous function of *NOTCH* signaling remains poorly understood. For this reason, we studied this dual activity of *NOTCH1* in arsenic-induced keratinocyte transformation, thus providing a model to investigate the molecular aspects determining whether *NOTCH* signaling will be either oncogenic or tumor-suppressive (12). We observed that the mechanism is characterized by two phases. The first phase involves the down-modulation of NOTCH1 expression, and the second phase involves the acquisition of resistance to arsenite-induced down-regulation of NOTCH1 (12). We found that maintenance of NOTCH1 expression supports metabolic activities to enhance cytoprotection against oxidative stress that as a side effect may sustain cell proliferation and keratinocyte transformation, strengthening the hypothesis that tumor cell selection could favor

This work was supported by Italian Association for Cancer Research (AIRC) Grant IG-15218, Ministero dell'Istruzione, dell'Università e della Ricerca Grant PRIN-2017XCXAFZ, and Sapienza-Progetti Ateneo 2017 (to C. T.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Table S1 and Figs. S1–S6.

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[4] The abbreviations used are: PS, presenilin; ICD, intracellular domain; HNSCC, head and neck squamous cell carcinoma; PLK1, Polo-like kinase 1; GSI, γ-secretase inhibitor; pH3, phosphorylated histone H3; mut, mutant; ERK, extracellular signal–regulated kinase.

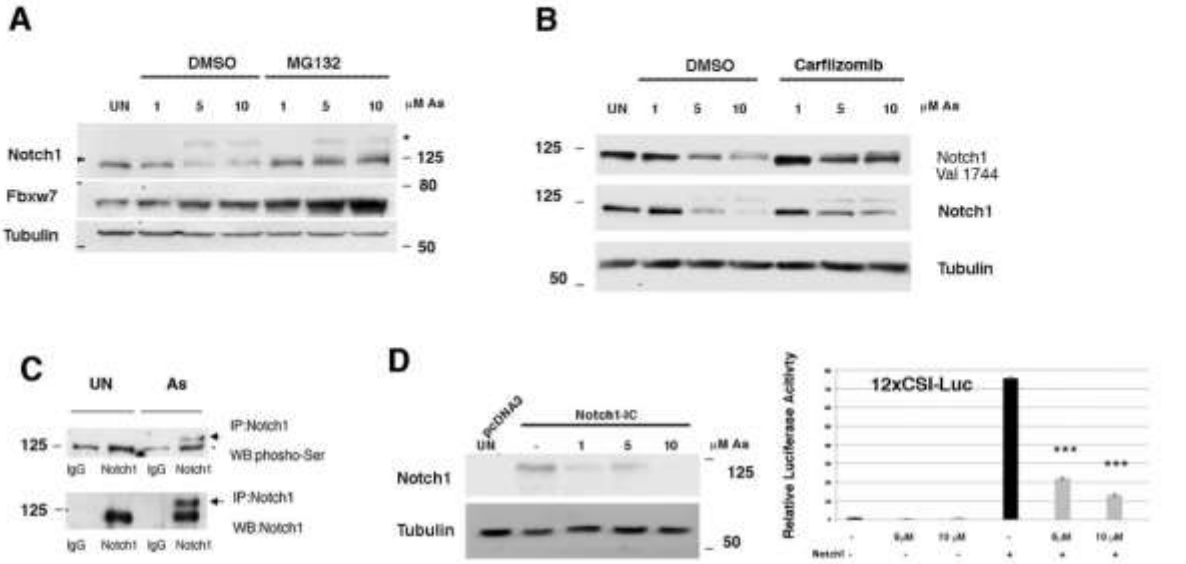

Figure 1. Decreased NOTCH1 levels in As_2O_3-treated keratinocytes. *A* and *B*, HaCaT cells were untreated or treated with As_2O_3 (*As*). 24 h post-treatment, cells were either untreated or treated with MG132/carfizomib for 5 h before collection; immunoblotting was performed with the indicated antibodies. *C*, HaCaT cells were treated with As_2O_3 for 24 h before collection, cell extract was immunoprecipitated (*IP*) using an antibody against NOTCH1, and immunoblotting (*WB*) was performed with the indicated antibodies. *D*, HaCaT cells were transfected with either pCDNA3 or NOTCH1-IC (encoding the human Notch1-IC, 1757–2555). 36 h post-transfection, cells were treated with As_2O_3 for 24 h before collection; immunoblotting was performed with the indicated antibodies. *D* (*right*), HaCaT cells were co-transfected with the *NOTCH*-responsive promoter 12xCSL, and the NOTCH1-IC plasmid was then treated with increasing amounts of As_2O_3 (5 and 10 μM) 12 h before collection. Average and S.D. values were calculated from triplicate samples. ***, $p < 0.0001$.

partial rather than complete inactivation of this signaling pathway (12). To identify regulators that may influence the dichotomous *NOTCH1* function, we screened a chemical library targeting human kinases and identified Polo-like kinase 1 (PLK1) as one of the kinases involved in arsenite-induced down-modulation of NOTCH1 expression. The Polo-like kinase is an important regulator of cell division responsible for a wide number of functions: centrosome maturation, DNA replication, mitotic entry, and adaptation to persistent DNA damage (13, 14). We identified NOTCH1 as a novel direct target of PLK1 kinase activity. *PLK1* inhibition reduced arsenite-induced NOTCH1 down-modulation. Arsenic is known to have genotoxic and mutagenic effects; genotoxic stress causes proliferating cells to activate the DNA damage checkpoint to assist DNA damage recovery by slowing cell cycle progression. Thus, to drive proliferation and transformation, cells must tolerate DNA damage and suppress the checkpoint response (see Ref. 15) and references therein). We report here that PLK1 promotes NOTCH1 down-modulation to the G_2-M transition; conversely, NOTCH1 remains active during a DNA damage–induced G_2 arrest. Our data show that NOTCH1 has pleiotropic effects in DNA damage-arrested cells, and also in those contexts where *NOTCH1* is known to play a tumor suppressor function, cancer cells might still be dependent on specific NOTCH1 signals to sustain their cancerous phenotype.

Results

PLK1 as a central kinase involved in arsenite-induced NOTCH1 down-modulation

To explore the mechanisms that determine whether NOTCH signaling will be either oncogenic or tumor-suppressive, we used a well-defined *in vitro* model in which the nontumorigenic human keratinocyte cell line (HaCaT) was acutely exposed to arsenic trioxide (arsenite). We previously demonstrated that loss of FBXW7 induction might contribute to acquire both resistance to arsenite-induced down-modulation of NOTCH1 and HaCaT transformation (12). Here we show that arsenite stimulates the serine phosphorylation of NOTCH1 with the parallel decreased expression of NOTCH1 and up-regulation of FBXW7 levels (Fig. 1, *A–C*). Treatment of cells with the proteasome inhibitors prevented the decrease of NOTCH1 expression (Fig. 1, *A* and *B*). FBXW7 is a constituent of the SCF ubiquitin ligase complex (SKP1-CUL1-F box) that controls the degradation of NOTCH1. Substrate phosphorylation is required for FBXW7-mediated recognition (16–18). Thus, we developed a luciferase assay to identify the kinase that would prime NOTCH1 for recognition by FBXW7. First, HaCaT cells were transiently transfected with an expression vector of NOTCH1-IC. At 36 h after transfection, the cells were treated with arsenite for the last 12 h at the indicated concentrations (1, 5, and 10 μM). Total cell lysates were collected and subjected to Western blot analysis. Arsenite treatment decreased the NOTCH1 level compared with the vehicle-treated control cells (Fig. 1*D*), indicating that exogenous NOTCH1-IC is degraded similarly to the endogenous NOTCH1. Then we used a 12xCSL-luciferase reporter vector responsive to *NOTCH1* signaling, and we found that NOTCH1 transcriptional activity was strongly suppressed by arsenite treatment (Fig. 1*D*, *right*). This functional assay was used to screen a kinase inhibitor library of 378 small-molecule compounds. All compounds were screened in triplicate at 10 μM in the presence of 5 μM arsenite (data not shown). Those compounds showing at least a >50% recovery of luciferase activity were further tested by luciferase assay and Western blotting (Figs. S1 and S2). We identified 27 kinases able to rescue the NOTCH1 luciferase

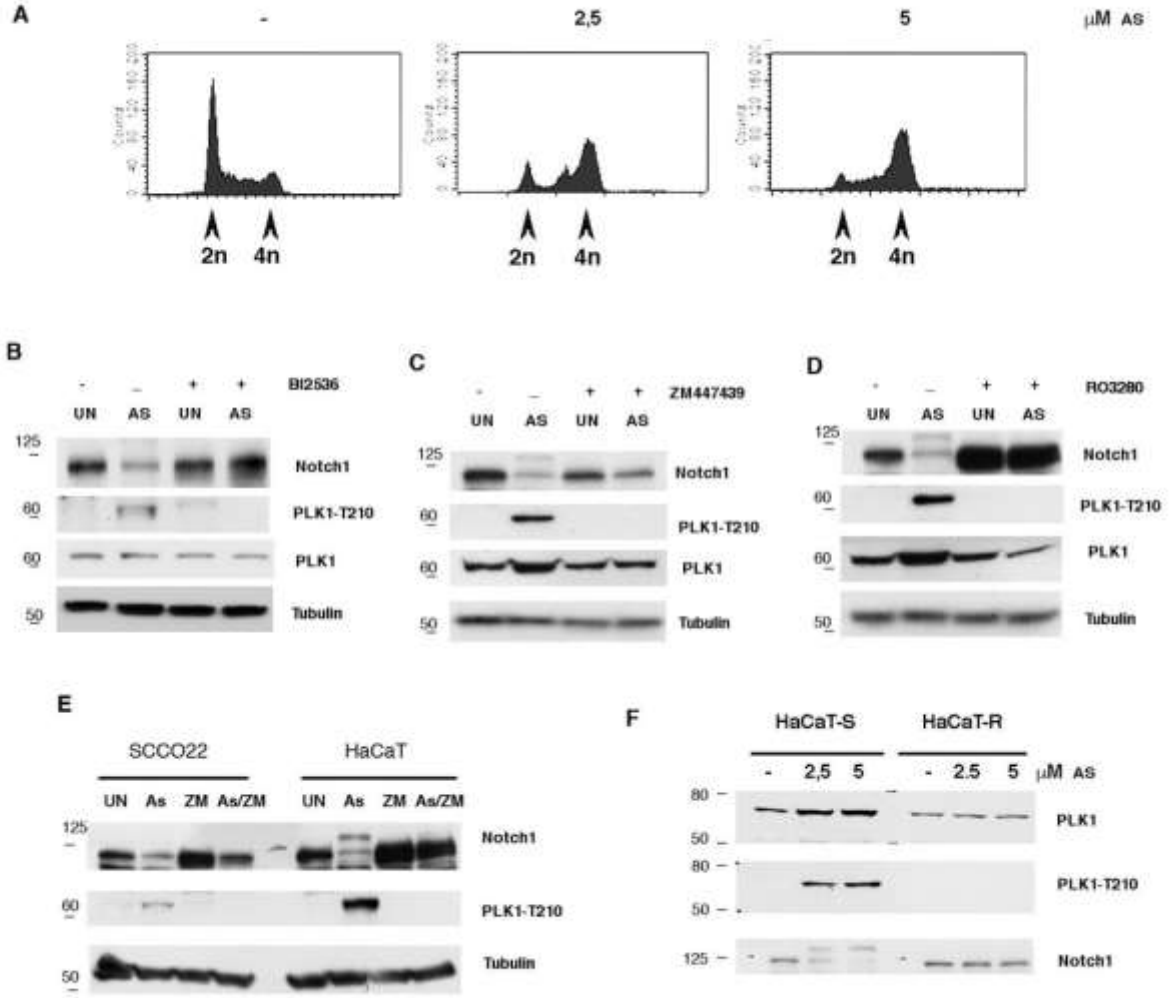

Figure 2. Effects of PLK1 inhibition in As_2O_3-treated cells. *A*, HaCaT cells were treated for 24 h with the indicated amount of arsenite, and then cells were collected, and the cell cycle was analyzed by FACS. *B–D*, immortalized HaCaT cells were treated with the indicated amount of As_2O_3 (*AS*) for 24 h, and then cells were treated with/without the indicated inhibitors (PLK1 inhibitor BI2536; ZM447439 Aurora A/B; RO3280 PLK1) for 24 h and analyzed by immunoblotting with the indicated antibodies. *E*, the indicated cell lines were treated with As_2O_3 for 24 h, and then cells were treated with/without 10 μM ZM447439 (*ZM*) for 24 h and analyzed by immunoblotting with the indicated antibodies. *F*, immortalized (HaCaT-S) and As_2O_3-transformed HaCaT cells (HaCaT-R) were treated with increasing amounts of As_2O_3 for 24 h and analyzed by immunoblotting with the indicated antibodies. Shown are representative results from at least three independent experiments. UN, untreated.

activity (Fig. S1). To understand the functional context of how the identified kinases might have an impact on NOTCH1, we performed a network analysis in which we investigated all possible direct and indirect interactions among them. For this purpose, the full Pathway Commons database of reported protein interactions in Simple Interaction Format (SIF) was performed. This analysis resulted in a network comprising 611 proteins with 2263 interactions (Fig. S3). The central component of the shortest path network was the protein PLK1. PLK1 is a promitotic kinase, and its main function is to facilitate the mitotic process (13, 14). However, PLK1 also promotes cell cycle progression in cells under stress conditions, thus facilitating tolerance to genotoxic stress (15). Arsenic is known to have genotoxic and mutagenic effects, and we observed that arsenite-treated cells were arrested in G_2 (12) (Fig. 2*A*). Thus, we tested whether PLK1 activity might affect NOTCH1 expression following arsenite treatment. PLK1 activation requires phosphorylation on a conserved threonine in the T-loop of the kinase domain (Thr-210). PLK1 is first phosphorylated on Thr-210 in G_2 phase by the kinase Aurora-A, in concert with its cofactor Bora (19, 20). Thus, to further characterize the pattern of Thr-210 phosphorylation and NOTCH1 stability, HaCaT cells were treated with arsenite and cultured in the presence or absence of both PLK1 and Aurora inhibitors. In agreement with the luciferase assay, accumulation of NOTCH1 protein upon treatment with PLK1 inhibitors was observed in arsenite-untreated and -treated HaCaT cultures as well as in SCC022, a squamous cell carcinoma–derived cell line (Fig. 2, *B*, *D*, and *E*). We previously demonstrated that arsenite-transformed keratinocytes acquire resistance to arsenite-induced NOTCH1 down modulation. Here, we

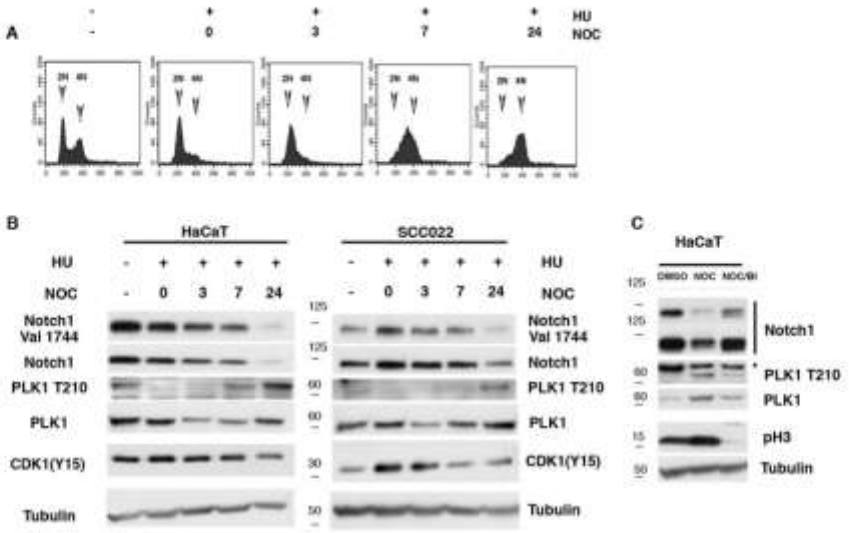

Figure 3. PLK1-dependent degradation of NOTCH1 at the G_2-M transition. *A* and *B*, HaCaT and SCC022 cells were collected at the indicated time points after release from G_1/S, cell cycle was analyzed by FACS (FACS profile is shown only for HaCaT cells), and cell lysates were immunoblotted with antibodies to the indicated proteins. *C*, HaCaT cells were treated for 16 h with nocodazole to induce a mitotic block, and BI2536 (PLK1 inhibitor) was added 8 h before harvesting. Prometaphase cells were then collected by shake-off, and cell extracts were analyzed by immunoblotting with antibodies to the indicated proteins. Shown are representative results from at least three independent experiments. *NOC*, nocodazole; HU, hydroxyurea.

observed PLK1 activation and NOTCH1 down-regulation after arsenite treatment in the presence of DNA damage signals, as shown by increased γ-H2AX (Fig. S5). We also found that PLK1 activation was not observed in arsenite-transformed keratinocytes (HaCaT-R) after arsenite treatment (Fig. 2*F*). This indicates that PLK1 activity might make a potential contribution at the early stages of arsenite carcinogenesis and that in arsenite-transformed keratinocytes PLK1 is no longer required in response to arsenite treatment, as cells have acquired a molecular switch required for cellular adaptation to genotoxic stress (*e.g.* metabolic adaptation) (12).

NOTCH1 is a direct target of PLK1

Analysis of the NOTCH1 C-terminal primary amino acid sequence by different computational platforms revealed the presence of multiple potential phosphorylation sites for the PLK1 consensus sequences (*RXX*(pS/pT)*XRXXR*). However, to narrow down the number of candidate motifs prior to experimental verification, we analyzed the NOTCH1 protein sequence by considering as putative candidate motifs only those identified via a high-stringency analysis and that can be recognized by both the PhoshoNET and GPS-Polo 1.0 platforms. Two sites, Ser-1791 and Ser-2349, were identified by these criteria (Fig. S4, *A–C*). Interestingly, both motifs are conserved across species, and Ser-1791 was found to be phosphorylated also in colon cancer cells (21). To confirm that NOTCH1 can be phosphorylated by PLK1, we performed an *in vitro* kinase assay using purified recombinant PLK1 and NOTCH1-IC fragment as substrate. As shown in Fig. S4*D*, the C-terminal NOTCH1 fragment was readily phosphorylated by PLK1. Additionally, when the two putative phosphorylation sites, Ser-1791 and Ser-2349, were replaced by Ala, WT NOTCH1-IC but not the mutant was efficiently phosphorylated (Fig. S4*E*).

To test whether the phosphorylation of NOTCH1-IC on the putative PLK1 phosphorylation sites determined the stability of NOTCH1-ICD cells expressing either WT NOTCH1-IC or mutants, NOTCH1-IC-A1791/A2349 constructs were treated with cycloheximide. At various time points thereafter, the transfected cells were lysed, and the amounts of the NOTCH1 proteins were measured by Western blot analysis. We found that mutation of Ser-1791/2349 promotes NOTCH1-IC stabilization (Fig. S4*F*).

NOTCH1 is a substrate of PLK1 in the G_2 phase of the cell cycle

To understand the functional significance of PLK1-mediated regulation of NOTCH1, we focused our attention to the PLK1/NOTCH1 expression during the cell cycle. It is well-known that in G_2, PLK1 is activated to promote entry into mitosis (see Ref. 14 and references therein). Thus, we sought to find the physiological conditions required to degrade NOTCH1 in the cell cycle context. To this purpose, we conducted synchronization experiments in HaCaT and SCCO22 human cells. A hydroxyurea block and release was performed to synchronize the cells in G_1/S, and the cell cycle profile was monitored. After the cells were released from the hydroxyurea-induced G_1/S block, the cells were harvested and subjected to a Western blot analysis. The phosphorylation of Thr-210 was observed strongly at the G_2 phase of the cell cycle, a pattern inversely correlated with the NOTCH1 expression (Fig. 3, *A* and *B*). However, the inhibition of PLK1 by BI2536 induced the accumulation of NOTCH1 protein (Fig. 3*C*), confirming that PLK1 promotes NOTCH1 down-modulation during the cell cycle. Our data indicate that PLK1 phosphorylates and consequently destabilizes NOTCH1 in the G_2-M transition. However, to be transformed, in cells under genotoxic stress, the checkpoint response should be down-regulated to tolerate the cellular DNA damage stresses. PLK1 activation regulates the checkpoint activation and allows cells to grow under genotoxic stress (22). Moreover, PLK1 is also known to be involved in promoting resistance to chemotherapeutic regimens with drugs such as doxorubicin (a

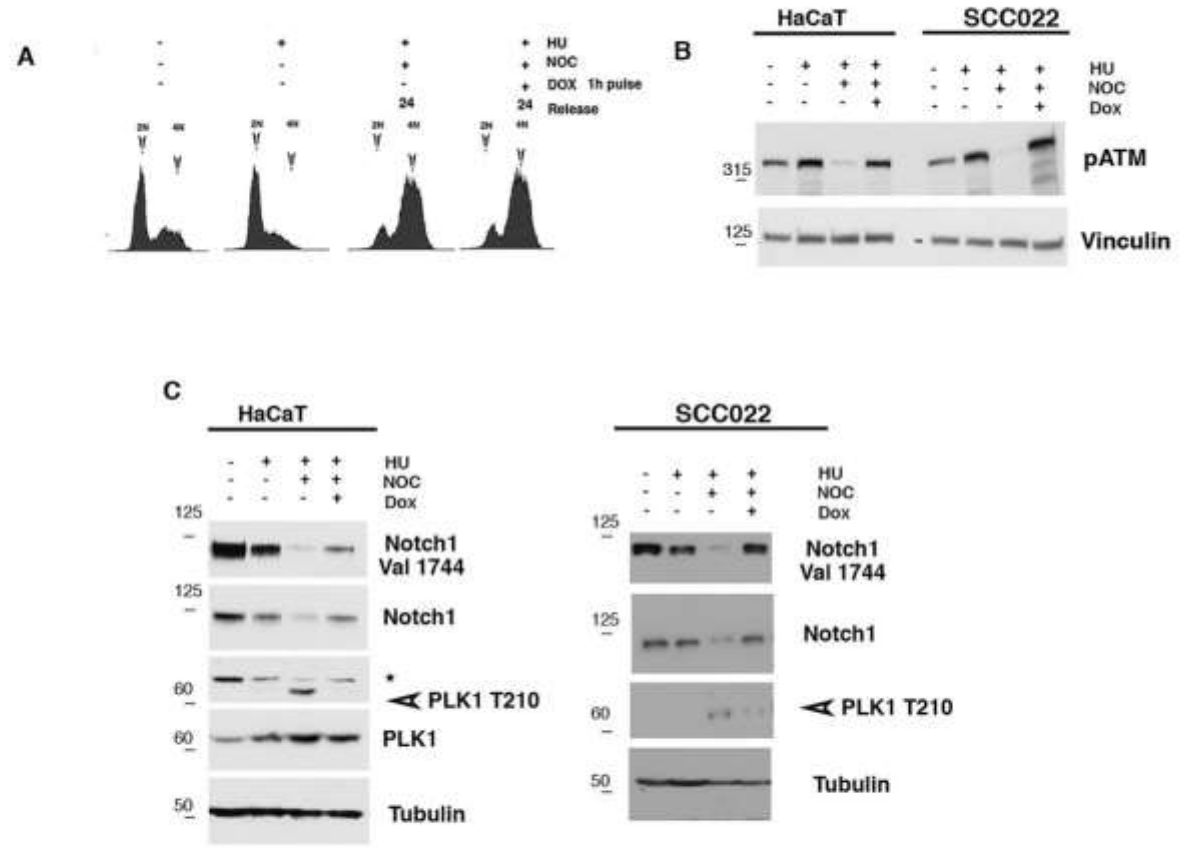

Figure 4. NOTCH1 expression in G_2 DNA damage arrest. *A–C*, HaCaT cells were left untreated (diagram 1) or treated with hydroxyurea (*HU*) for 19 h (*A*). Alternatively, cells were released from the hydroxyurea block and either untreated or treated after 7 h with doxorubicin (*DOX*) for 1 h and subsequently grown in the presence of nocodazole (*NOC*) for 18 h. Following these treatments, cells were collected at the indicated time points after release from G_1/S, cell cycle was analyzed by FACS (FACS profile is shown only for HaCaT cells), and cell lysates were immunoblotted with antibodies against the indicated proteins (*B* and *C*). Shown are representative results from at least three independent experiments.

DNA-intercalating compound) (23). We found that under arsenite treatment, NOTCH1 is continuously degraded, and in this condition, PLK1 is active (Figs. 1 and 2). Notably, a G_2 phase–specific inactivation of PLK1 after DNA damage has been described. The reason for this inactivation is to promote cell cycle exit to avoid proliferation and entry in mitosis in the presence of damaged DNA. Thus, we investigated whether PLK1 targets NOTCH1 during G_2 in response to DNA damage. To this end, both HaCaT and SCCO22 cells were synchronized at G_1/S and then allowed to progress through the cell cycle. At 7 h after the release from G_1/S (when cells were in G_2), cells were pulsed with doxorubicin for 1 h to induce DNA damage and harvested 18 h after doxorubicin release (Fig. 4*A*; only HaCaT cells are shown). As expected, induction of DNA damage results in decreased levels of PLK1 and activation of ATM (Fig. 4, *B* and *C*). Notably, when PLK1 was dephosphorylated and inactive, the expression of NOTCH1 was restored, indicating that NOTCH1 expression is up-regulated during the G_2 damage checkpoint (Fig. 4, *B* and *C*). Interestingly, similar results were obtained in FaDu cells, a SCC cell line with mutated p53, and HeLa cells, an adenocarcinoma cell line with WT p53 (Fig. S6), strengthening the argument that NOTCH1 and PLK1 are inversely correlated during DNA damage response.

Upon DNA damage in G_2, NOTCH1 protects cells from apoptosis

To unravel how PLK1 and NOTCH1 might functionally interact, we investigated whether NOTCH1 had a mitotic role. To this end, we made use of Ser-1791/2349 mutant NOTCH1-IC. SCCO22 cells were transfected with either empty vector or NOTCH1-IC Ser-1791/2349 mutant. Cells were synchronized at the G_1/S and released into the cell cycle; we did not observe any difference in cell cycle progression as phosphorylated histone H3 (pH3) showed the same kinetic during release (Fig. 5*A*) in both control– and NOTCH1-IC Ser-1791/2349 mutant–treated cells. Furthermore, no mitotic delay was detected in cells examined at either early time (1 and 2 h) or at longer times after nocodazole release (Fig. 5*B* and data not shown). We conclude that in this cellular context, NOTCH1 does not alter the G_2/M transition. Previous observations established that PLK1 plays a critical role in the G_2 checkpoint recovery following DNA damage (14, 24), and we found that NOTCH1 expression is up-regulated during the G_2 damage checkpoint (Fig. 4). Thus,

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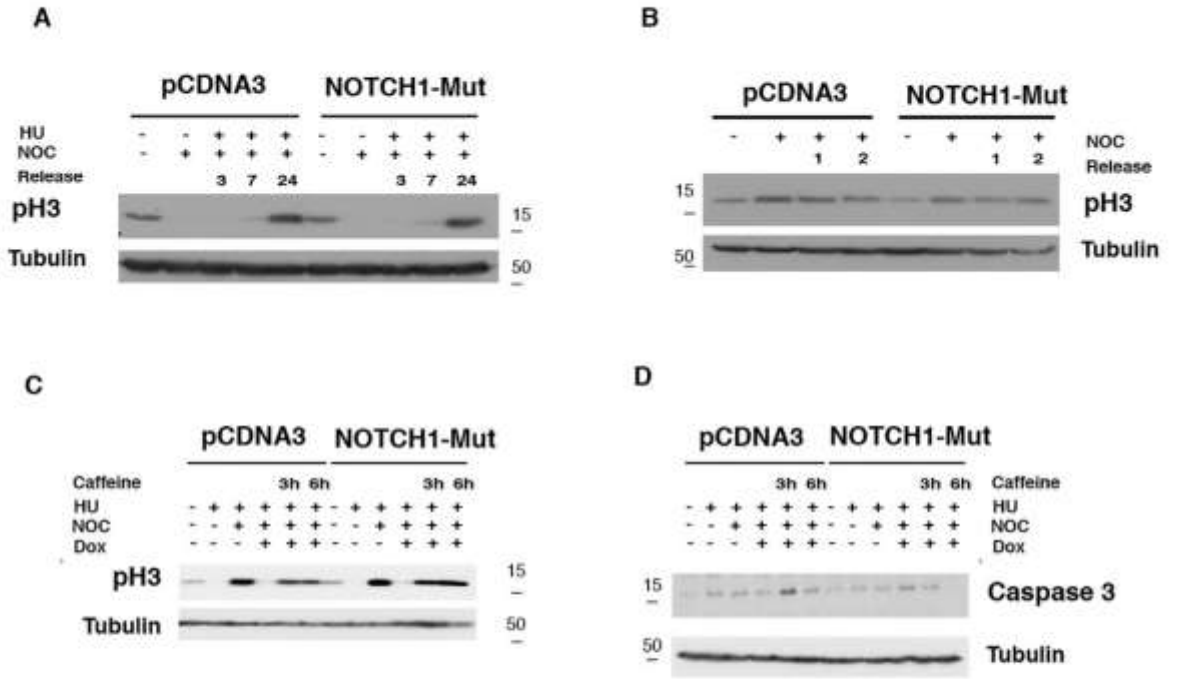

Figure 5. Overexpression of NOTCH1 mutant unphosphorylable by PLK1 has no effect on cell cycle progression. *A*, SCCO22 cells were transfected with either control, empty-PCDNA3 vector, or A1791/A2391-NOTCH1-ICD mutant. The cells were synchronized with hydroxyurea (*HU*) for 19 h. At the indicated time points after release, the cells were harvested and subjected to immunoblotting for the indicated proteins. *B*, cells were treated as described for *A*, except that cells were trapped with nocodazole (*NOC*) for 14 h and then released. At the indicated time points after release, the cells were harvested and analyzed with the indicated antibodies. *C* and *D*, SCCO22 cells were transfected with either control, empty-PCDNA3 vector, or A1791/A2391-NOTCH1-ICD mutant. The cells were synchronized with hydroxyurea for 19 h. Cells were released from the hydroxyurea (HU) block and either untreated or treated after 7 h with doxorubicin for 1 h and subsequently grown in the presence of nocodazole and caffeine the last 3 and 6 h. Cells were harvested and subjected to immunoblotting for the indicated proteins.

we evaluated whether NOTCH1 expression would alter recovery following DNA damage. To test this, cells were synchronized at the G_1/S and released into the cell cycle. After 6 h from release, cells were treated with doxorubicin to induce the G_2 damage checkpoint. Later cells were treated with caffeine to abrogate the G_2 checkpoint response. As expected, we detected an increase of pH3 in empty vector–treated cells after caffeine addition (Fig. 5*C*). Interestingly, NOTCH1-IC mut expression enhanced pH3 expression (Fig. 5*C*). Treatment of cells with caffeine abrogates the G_2 checkpoint but also promotes mitotic catastrophe and apoptosis (14). Consistently, we found that in empty vector–treated cells, caffeine treatment induced activation of caspase-3, whose expression levels were reduced in NOTCH-IC mut–treated cells (Fig. 5*D*). Although we observed a differential expression of the cleaved caspase-3, neither empty vector– nor NOTCH1-IC mutant–treated cells showed sign of apoptosis after the caffeine addition (data not shown). The mechanism by which DNA-damaged cells escape from apoptosis during the DNA damage checkpoint is poorly understood. Therefore, we wondered whether the requirement of NOTCH1 during the DNA damage–induced G_2 checkpoint could be restricted to such an anti-apoptotic signaling. To test this, we designed an experimental set-up to examine whether a cell cycle arrest/restart following a DNA damage–induced G_2 arrest in HaCaT cells would be dependent on the function of NOTCH1. HaCaT immortalized cells were chosen because in this cellular context, conversely to SCCO22 cells, sustained DNA damage checkpoint promotes apoptosis. Thus, HaCaT cells released from a hydroxyurea block were treated with doxorubicin at 7 h after release, a time at which the great majority of the cells had completed S phase (Fig. 6*A*). Using this approach, we were able to obtain a highly synchronous population of cells arrested at the G_2 DNA damage checkpoint by doxorubicin (Fig. 6*A*). Subsequently, we mimicked checkpoint silencing by the addition of the checkpoint kinase inhibitor caffeine and allowed the cells to enter mitosis in the presence of nocodazole. Notably, doxorubicin treatment of HaCaT cells resulted in lower mitotic index when compared with control cells (Fig. 6*A*, *bottom panels*, *diagrams 3* and *4*). After 3–6 h of caffeine treatment, a significant fraction of cells entered mitosis as judged from phosphohistone H3 staining (Fig. 6*A*, *bottom panels*). When cells entering in the G_2 damage–induced checkpoint were examined in more detail, a decrease in pPLK1 level and the appearance of NOTCH1 expression were observed (Fig. 6*B*, *lanes 3* and *4*). When we analyzed cell recovery from DNA damage–induced arrest after doxorubicin treatment, we found that G_2-arrested cells could be forced to enter mitosis following the addition of caffeine. Interestingly, caffeine treatment increased PLK1 expression, indicating that, as previously shown, PLK1 becomes essential for mitotic entry and recovery from a DNA damage–induced G_2 arrest (24). Consistent with a role for PLK1 in the control of NOTCH1 expression, we found

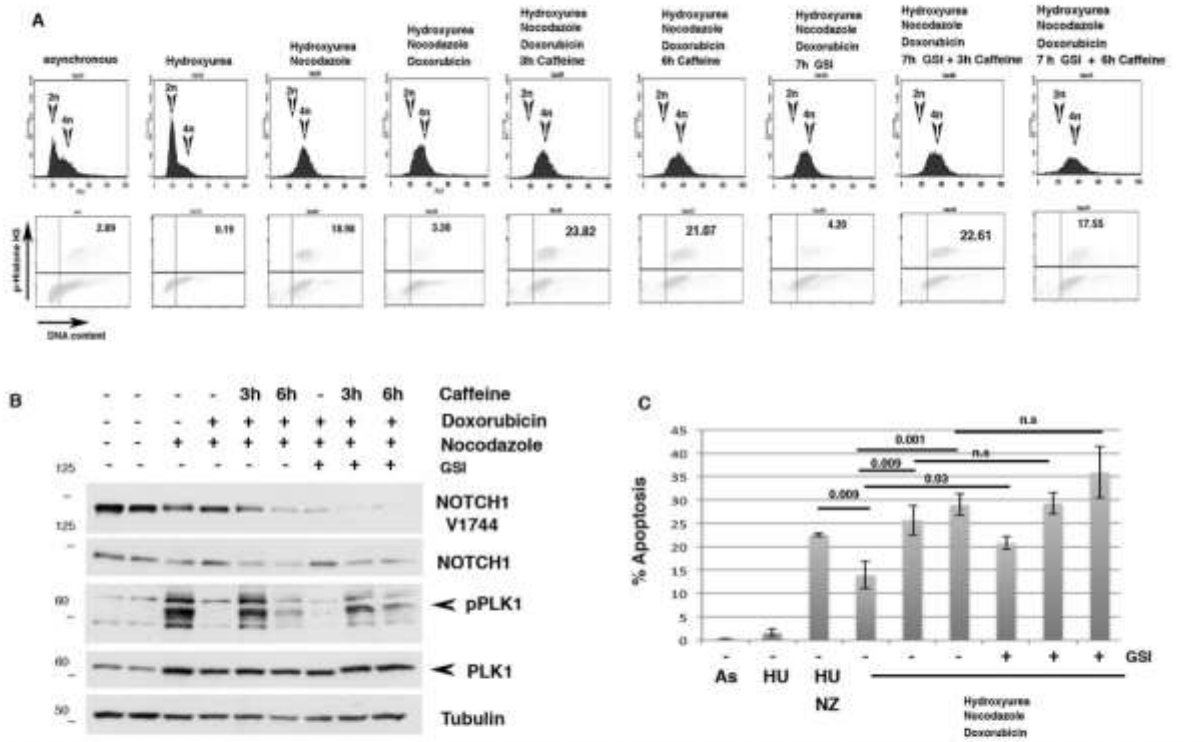

Figure 6. NOTCH1 expression in recovery from a G_2 DNA damage arrest. *A*, HaCaT cells were left untreated or treated with hydroxyurea (*HU*) for 19 h. Alternatively, cells were released after the hydroxyurea block and, 7 h after release, treated with doxorubicin for 1 h and subsequently grown in the presence of nocodazole for 18 h. Following these treatments, caffeine was added for the indicated time periods to allow recovery from the checkpoint-induced arrest 3 and 6 h before harvesting the cells. DNA content and phosphohistone H3 positivity were determined. *B*, cells were treated as described in *A*, and whole-cell lysate was used for Western blotting with the indicated antibodies (*C*). Cells were treated as described in *A*, and percentage of apoptosis was determined by FACS analysis. *n.s.*, not significant; *As*, As_2O_3; *NZ*, nocodazole.

that pPLK1 activation was paralleled by NOTCH1 down-modulation when caffeine was added to induce recovery from a DNA damage–induced G_2 arrest (Fig. 6*B*). Notably, NOTCH1 does not seem to be instrumental for achieving a DNA damage–induced arrest, because GSI-treated cells efficiently arrested in response to DNA damage (Fig. 6*A*, *seventh diagram*). Strikingly, when we examined the fate of the damaged cells that are in the DNA damage–induced G_2 arrest or induced to enter mitosis by the addition of caffeine in the presence of GSI, we found that cell viability was severely affected (Fig. 6*C*). These results demonstrate that NOTCH1 protects cells from DNA damage–induced arrest and that PLK1-mediated degradation of NOTCH1 may be essential for recovery from a DNA damage-induced arrest.

NOTCH1 promotes inflammatory cytokine secretion in cancer cells that undergo growth arrest in response to DNA damage

Induction of cell cycle arrest in response to DNA damage represents a protective mechanism against harmful mutations but also promotes apoptosis (14, 24). We found that NOTCH signaling protects immortalized HaCaT cells from DNA damage–induced apoptosis. Conversely, we observed that in the squamous cell carcinoma cell line, SCCO22, induction of DNA damage by doxorubicin treatment promotes a permanent cell cycle arrest with no sign of apoptosis (Fig. 7 and data not shown). In response to DNA damage, growth-arrested cancer cells also develop a secretory phenotype that alters tissue microenvironments and might stimulate tumor growth *in vivo* (25). Among the secreted factors, IL-6 and IL-8 are of particular interest. These cytokines have been shown to promote tumorigenesis by regulating processes associated with tumorigenesis raging from cancer metabolism to metastasis (25, 26). Therefore, we wondered whether NOTCH1 during DNA damage–induced G_2 checkpoint could be involved in such secretory signaling. To test this, SCCO22 cells were treated with doxorubicin to induce the G_2 damage checkpoint (Fig. 7*A*). Later, cells were treated with GSI to inhibit NOCTH signaling (Fig. 7, *B* and *C*). As expected, we detected an increase of IL-6 and IL-8 in doxorubicin-treated cells (Fig. 7*C*). Interestingly, GSI treatment decreased both IL-6 and IL-8 expression (Fig. 7*C*) but not TGFB1 that has been associated with the development of a secretory phenotype of cancer cells. Thus, these data support a model in which the epithelia cancer cells, SCCO22, use Notch signaling to support a secretory phenotype.

Discussion

NOTCH1 activity plays pivotal roles in signaling for diverse cellular process, such as cell differentiation, stem cell renewal, proliferation, and transformation (1, 8, 27). *NOTCH1* signaling has been reported to have a contradictory role in cell transfor-

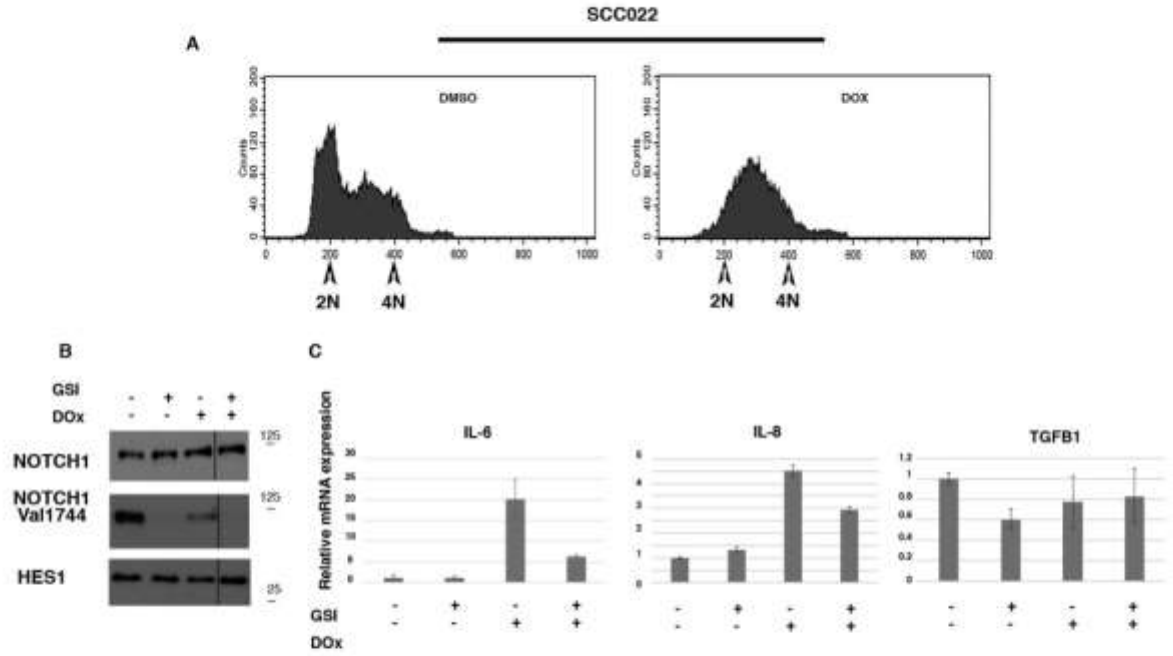

Figure 7. NOTCH1-dependent increased expression of IL-6 and IL-8 during DNA damage–induced growth arrest. SCC022 cells were treated with doxorubicin (*DOX*), following which either DMSO or GSI was added, and cells were maintained in culture for a further 24 h. In *A*, cells were analyzed by FACS analysis. *B*, cells were treated as described in *A*, and whole-cell lysate was used for Western blotting with the indicated antibodies. Additional samples present on the gel were cropped as indicated by *dashed lines*. In *C*, cells were treated as described for *A*, and total RNA was used for quantitative RT-PCR with the indicated probe.

mation (4, 8). However, a widely accepted model implies that the impact of *NOTCH1* signaling is highly context-dependent, and it can have opposite effects in different systems. We have used arsenite-induced malignant transformation of a human epithelial cell line as an *in vitro* model to study the mechanisms that can result in NOTCH1 role and function alterations (12). We previously demonstrated that whereas arsenite-mediated apoptosis of immortalized keratinocytes was associated with NOTCH1 down-regulation, arsenite-mediated transformation of these cells was characterized by increased NOTCH1 stability (12). We found that *NOTCH1* regulates cellular metabolism and apoptosis, which in turn differentially impact cell proliferation and cell transformation (12). Consequently, the cellular genetics/context may impinge on the antagonistic duality of *NOTCH1* function. We presented evidence indicating that *FBXW7* is required for the differential expression of NOTCH1 during arsenite-mediated transformation, indicating that kinases and biochemical pathways could be involved in NOTCH1 phosphorylation in tumors. Given that NOTCH1 stability and signaling are controlled by its phosphorylation (21), the study of kinases that could be implicated in this post-translational modification could help to elucidate the mechanisms controlling NOTCH1 dichotomy in cancer development. In this study, the effects of 378 cellular kinase inhibitors on NOTCH1 transcriptional activity and protein stability after arsenite treatment were investigated. Our findings indicate that multiple kinases implicated in various cell signaling pathways can participate in these outcomes: FAK, IKKB, PKA, ATM, ATR, SRC, p38, m-TOR, GSK1, c-MET, CDK1, ALK, PLK1, AURKA/B, CSF1R VEGFR, and JAK. To understand how the identified kinases might have an impact on NOTCH1, we performed a network analysis to investigate all possible direct and indirect interactions among them. The central component of the shortest path network was the protein PLK1, which is a central regulator of cell division required for several events of mitosis and cytokinesis (13, 14). Whereas in nondamaged cells, the PLK1 pathway is involved in G_2/M transition, PLK1 was shown to be a direct target of the G_2 DNA damage checkpoint. Indeed, in response to a wide range of DNA-damaging agents, PLK1 was shown to be catalytically inactivated. Moreover, this inhibition was shown to depend on functional ATM or ATR (14). Such control of the cell cycle machinery may be critically important to prevent a premature restart of the cell cycle following genotoxic stress. However, in addition to being a target of the DNA damage checkpoint, PLK1 was also shown to regulate cell cycle progression after a damage-induced cell cycle arrest. In this context, cells escape the DNA damage checkpoint arrest in a process called "adaptation." Such a mechanism allows damaged cells to eventually divide and possibly survive and undergo transformation (14, 15). Consistent with the above observation, we found that when challenged with arsenite, cells were G_2-arrested. The data presented here show that NOTCH1 is a novel substrate of PLK1. Additionally, we found that in an unperturbed cell cycle, PLK1 appears to be involved in NOTCH1 down-modulation at the mitotic entry. Interestingly, we observed an increase in the levels of Thr-210 PLK1 expression, which indicates that PLK1, by facilitating tolerance to arsenite-induced genotoxic stress, might favor arsenite-induce

cell transformation. Notably, the coordination of this pathway becomes critical for both DNA damage checkpoint and mitotic entry in cells recovering from a DNA damage–induced arrest (28). Although its exact involvement remains to be established, in arsenite-induced transformation, NOTCH1 represents a checkpoint mediator targeted by PLK1 to silence the DNA damage checkpoint in a condition in which damage persists for long periods of time. Thus, PLK1 activation initiates an escape program from checkpoint-mediated arrest prior to completion of damage repair. NOTCH1 inactivation is part of the PLK1-associated adaptation program to DNA damage that can result in enhanced cell death (*e.g.* through mitotic catastrophe) but at the same time may allow the propagation of defects in the genome to the daughter cells that may contribute to cell transformation. Although our observations necessitate further analysis to understand how deregulation of the NOTCH1 pathway impacts signaling that responds to DNA damage, we provide evidence that Notch signaling is altered but not abolished in SCC cells. We found that NOTCH signaling might contribute to the secretory phenotype of epithelial cancer cells. Thus, the dual role of Notch in cancer biology is undoubtedly complex and tumor type–independent. It is important to recognize that even in a single type of tumor, there is plasticity in Notch function that deserves greater attention.

Experimental procedures

Cell culture and transfection

HaCaT-S immortalized and HaCaT-R cells were described previously (12). Culture cells 70–80% confluent were maintained in modified low-calcium medium and transfected using the Lipofectamine transfection reagent (L-006119-00, Thermo Scientific/Dharmacon (Lafayette, CO)) according to the manufacturer's instructions (Thermo Fisher Scientific). Cells were analyzed at the indicated times after transfection by either RT-PCR analysis or Western blotting, as indicated (12, 29). SCCO22 were kindly provided by Dr. Caterina Missero (Università degli Studi di Napoli, Naples, Italy). HeLA and FaDu were kindly provided by Dr. Angelo Peschiaroli (CNR, Rome, Italy).

Reagents and immunoblotting

The following reagents were purchased from Santa Cruz Biotechnology: Fbxw7 and tubulin. In addition, we used Notch1 Val-1744, Notch1 D1E11, PLK1 208G4, and PLK1(Thr-210) from Cell Signaling Technology (Beverly, MA). γ-Secretase inhibitor IX (DAPT), was purchased from Calbiochem (Merck KGaA), dissolved in DMSO, and stored at −20 °C until use. All cell extracts were prepared as described previously (30) and according to the manufacturer's instructions for detection of phospho-ERK (Cell Signaling Technology). The kinase library of 378 structurally diverse, cell-permeable kinase inhibitors was purchased from Selleckchem (Houston, TX) (catalogue no. L1200) (Table S1).

Notch1-ICD encodes the expression of human Notch1-IC from amino acid 1757 to 2555 and has been described previously (9). GST-NOTCH1-IC plasmid encodes the GST-Notch1-IC fusion protein encoding the mouse NOTCH1-IC region 1753–2531 was kindly provided by Dr. Lendhal (Karolinska Institute, Stockholm, Sweden) and described previously (31). The plasmids containing mutations in Notch1-ICD encoding the expression of human Notch1-IC from amino acid 1757 to 2555 were generated using the QuikChange II XL site-directed mutagenesis kit (Thermo Fisher Scientific) and verified by sequencing.

Kinase library screening

Transient transfection/promoter activity assays were performed using a Dual-Luciferase/*Renilla* reporter assay system (Promega). All conditions were tested in triplicate samples, and a 12xCSL-luciferase reporter vector responsive to *NOTCH* signaling was co-transfected with either pcDNA3 as control or NOTCH1-IC vector. At 24 h after transfection, cells were treated with compounds in triplicate at 10 μM, and a luciferase assay was conducted in the presence of 5 μM arsenite. The results were normalized against *Renilla* luciferase. To control for cytotoxic effect of the compounds, when the *Renilla* luciferase activity was reduced to <25% of the activity seen with the vehicle-treated controls and the survival rate was less than 75%, those compounds were excluded from further analysis. Those compounds showing a ≥50% recovery of luciferase activity were further tested in increasing amounts. In this second step, each compound was tested in increasing amounts (1, 5, and 10 μM) in the presence of 5 μM arsenite. All compounds were further tested for their ability to rescue NOTCH1 expression after arsenite treatment by Western blotting at 10 μM in the presence/absence of 5 μM arsenite.

PLK1 kinase assay

For the PLK1 kinase assay, GST-Notch1 fusion protein was expressed in *Escherichia coli* BL21 strain and purified using standard procedures. PLK1 kinase assays were carried out using the PLK1 activity assay reagent kit purchased form SignalChem (Richmond, Canada) according to the manufacturer's instructions.

Cell cycle analysis

To analyze mitotic entry, cells were fixed and stained with propidium iodide and an antibody against phosphohistone H3 (Ser-10) using the FlowCellect™ cell cycle kit for G_2/M analysis (EMD Millipore, Darmstadt, Germany). The percentages of M-phase cells and cellular DNA content were determined by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Synchronization and recovery from DNA damage

HaCaT, SCCO22, FaDu, and HeLa cells were grown in Dulbecco's modified Eagle's medium and RPMI supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For the synchronization experiments, cells were incubated in hydroxyurea (1.5 mM) for 19 h to arrest cells at the G_1/S transition. Where indicated, the G_2/M DNA damage checkpoint was activated by treating cells with 0.5 μM doxorubicin for 1 h at 7 h after release from a hydroxyurea block. Doxorubicin was washed away thoroughly, and immediately after washing, nocodazole (250 ng/ml) was added to the culture medium. 18 h after washing away doxorubicin, all cells were arrested in G_2 as judged from FACS analysis. To inactivate DNA damage signaling and allow mitotic entry, caffeine (5 mM)

was added to inhibit ATR and ATM checkpoint kinases. The continuous presence of nocodazole prevented exit from mitosis and allowed accumulation of cells in mitosis. To inactivate NOTCH1 signaling, GSI (5 μM) was added 30 min before doxorubicin treatment and then maintained until cells were harvested for further analysis.

Author contributions—C. D. B. and C. T. formal analysis; C. D. B., A. Z., M. F., G. M., S. Cialfi, N. V., D. Benelli, and C. T. investigation; C. D. B., A. Z., and C. T. methodology; S. Checquolo, D. Bellavia, R. P., and I. S. resources; D. Benelli and C. T. conceptualization; C. T. data curation; C. T. funding acquisition; C. T. writing-original draft; C. T. project administration; C. T. writing-review and editing.

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PLK1 targets NOTCH1 during DNA damage and mitotic progression

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J. Biol. Chem. 2019, 294:17941-17950.
doi: 10.1074/jbc.RA119.009881 originally published online October 9, 2019

Supporting Information

Title:

PLK1 targets NOTCH1 during DNA damage and mitotic progression.

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S1

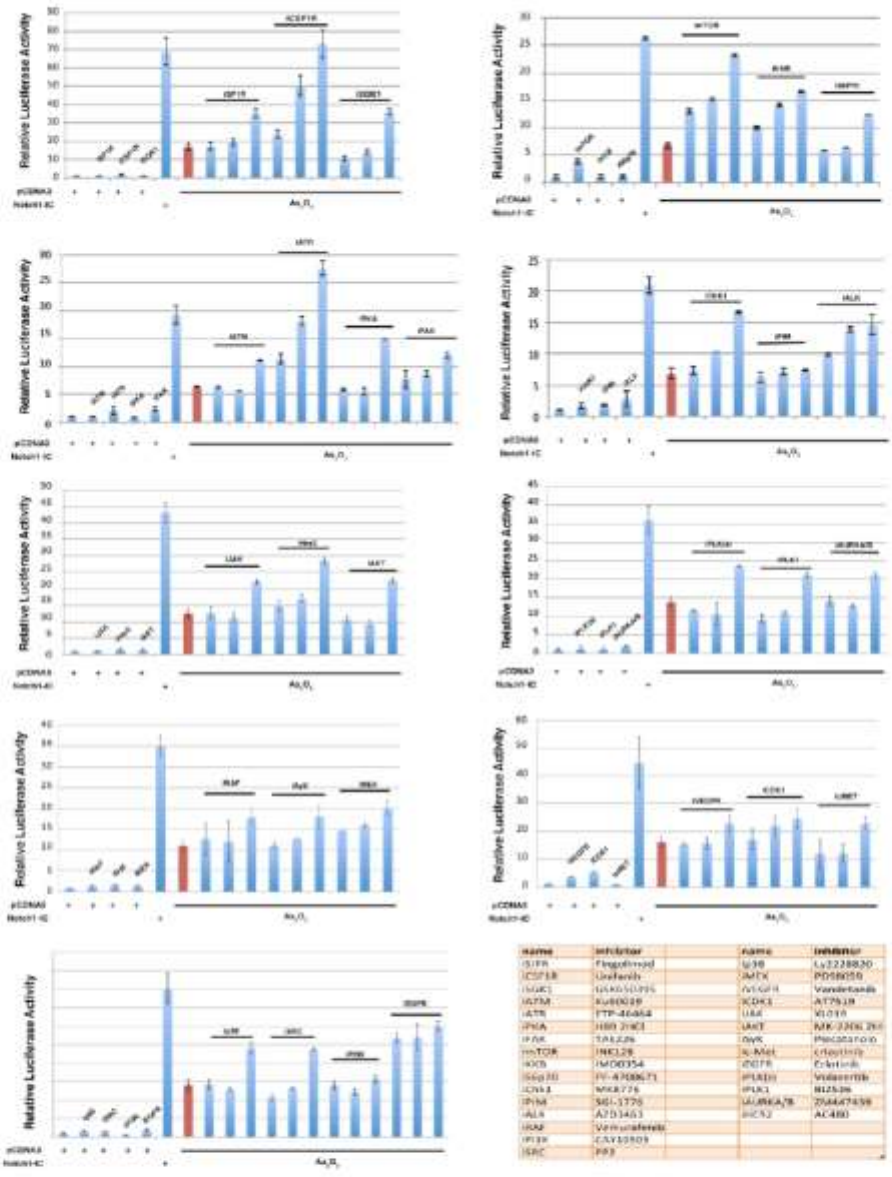

Fig.S1

Figure S1. A) HaCaT cells were co-transfected with the *NOTCH* responsive promoter 12XCLS and the NOTCH1-IC plasmid plus 5μM As_2O_3 and increasing amounts of the indicated inhibitors. 1, 5 10 μM. As control cells were co-transfected with the *NOTCH* responsive promoter 12XCLS and the pDNA3 plasmid plus 5μM of the indicated inhibitors.

S2

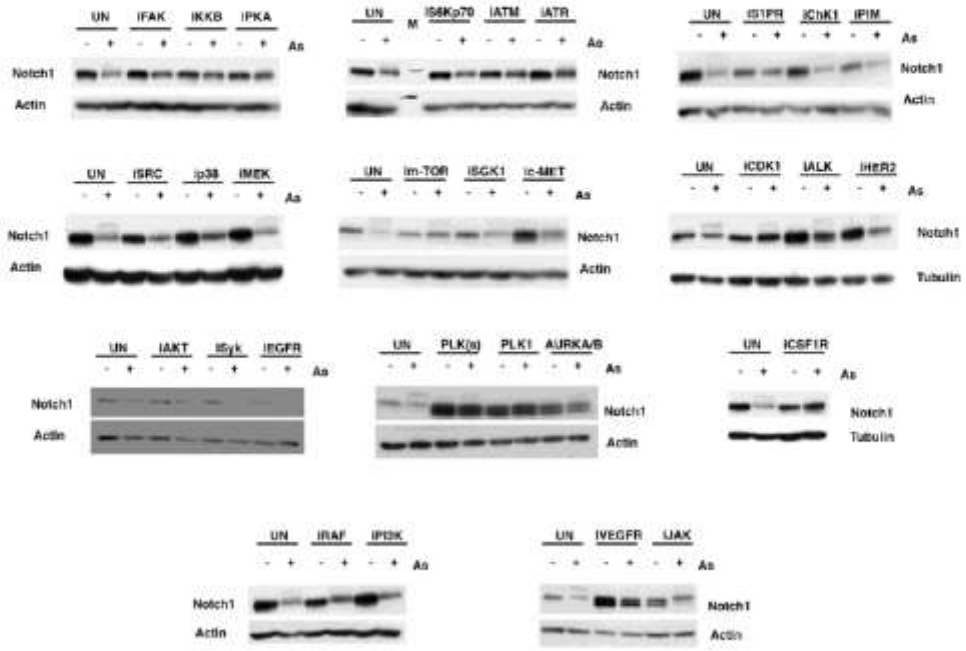

Fig.S2

Figure S2. A) HaCaT cells were treated with either DMSO or 5μM As_2O_3 alone or in combination with the indicated inhibitors (10μM). Cell extracts were analyzed by western blot.

S3

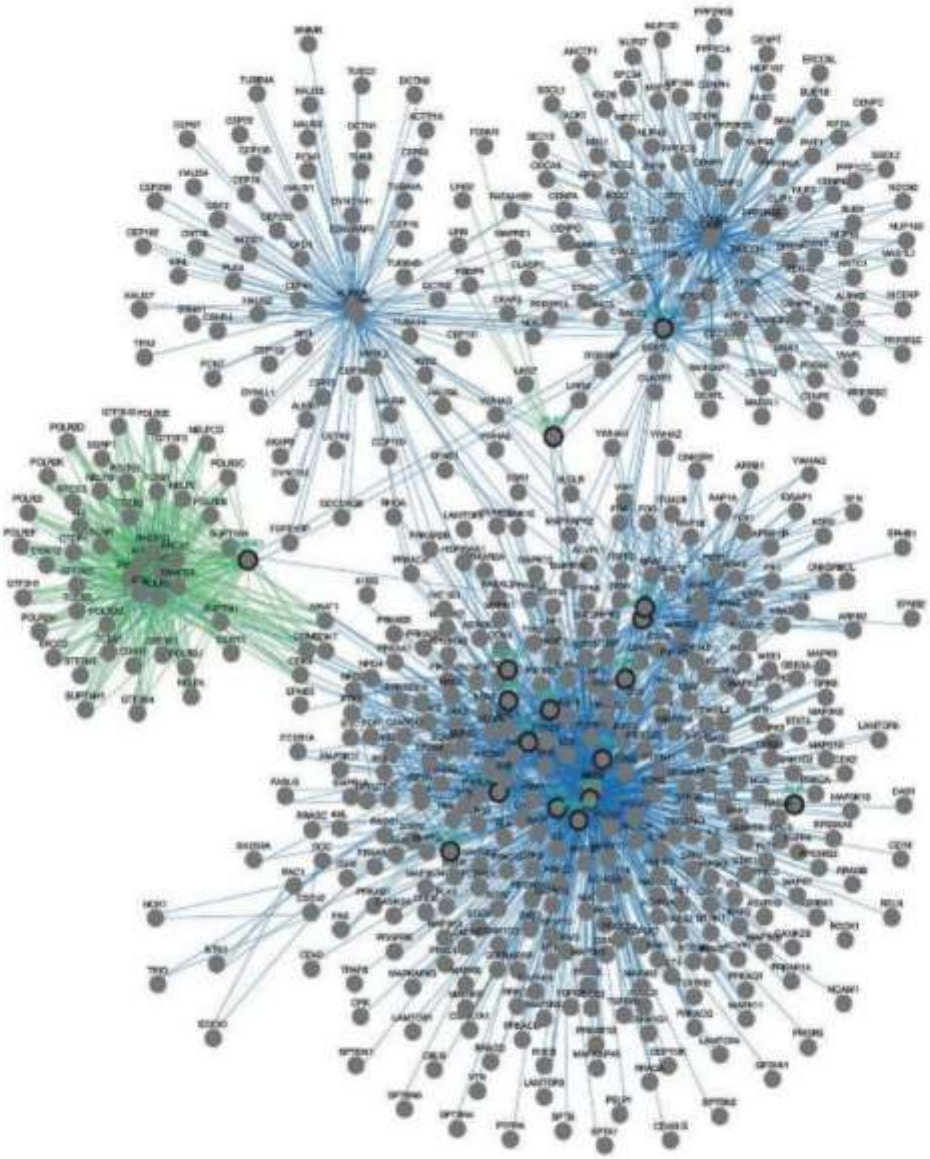

Figure S3. Pathway commons network visualizer (PCViz) (http://www.pathwaycommons.org) was used to detect functional interaction among the kinases identified in the screening experiments.

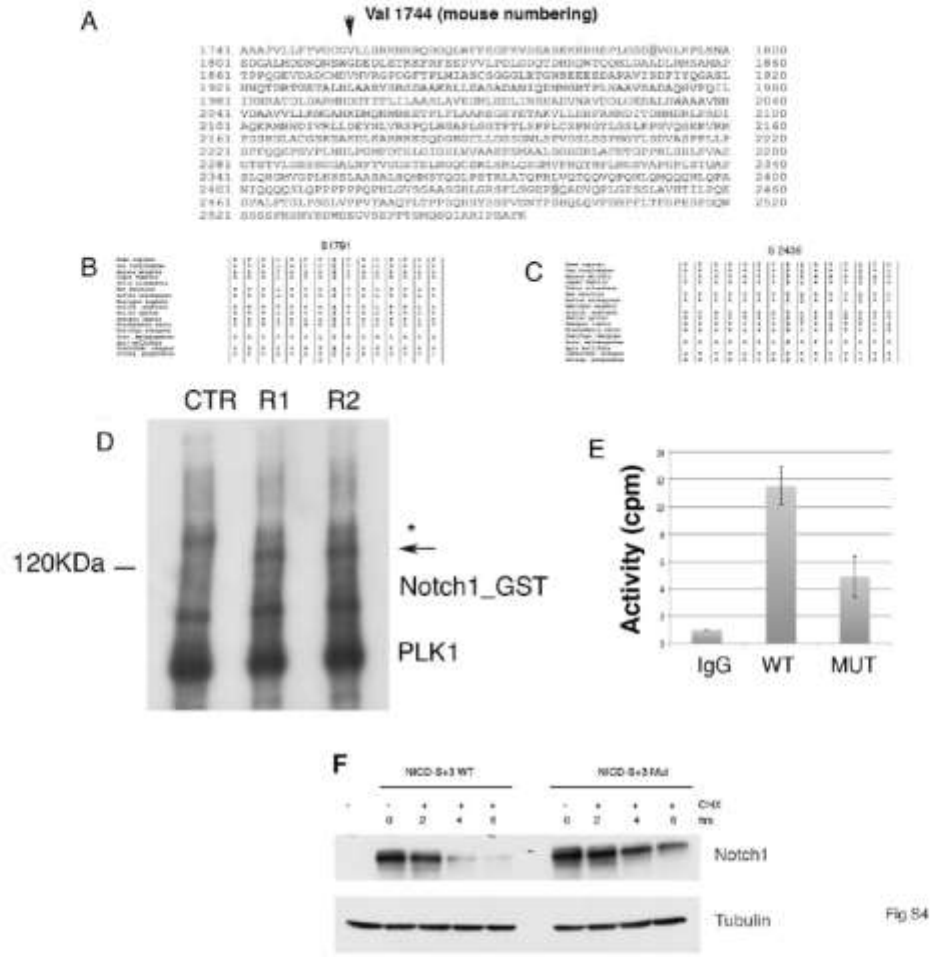

Figure S4. Direct In Vitro phosphorylation of Notch1 by PLK1.

A) Phosphorylation sites identified in NOTCH1-IC using KINEXUS and GPS-Polo 1.0 platform. (**B-C**) Evolutionary conservation of Ser1791 and Ser 2439 in the Notch1-IC sequence, analysis was performed by Kinexus platform (http://www.phosphonet.ca) (**D**) GST-NOTCH1-IC 1754-2555 was incubated with or without PLK1 followed by in vitro kinase assays. Fractions of the same reactions were analyzed by 7.5% SDS-PAGE. Experiment was performed in two replicates R1 and R2. **E)** NOTCH1-ICD was immunoprecipitated from HaCaT cells transfected with either WT and NOTCH1-IC mutant protein and analyzed by *in vitro* kinase assays as indicated in D except that the assay reaction was analysed by spotting the reaction mixture onto strips of P81 paper and analyzed in a scintillation counter. **F)** HaCaT cells were transfected with plasmids expressing WT-NOTCH1-ICD and mutant A1791/A2391-NOTCH1-ICD for 24 h, then treated with 100µm Cycloheximide for the indicated periods of time. The levels of NOTCH1 in the cell lysates were determined by Western blotting.

S5

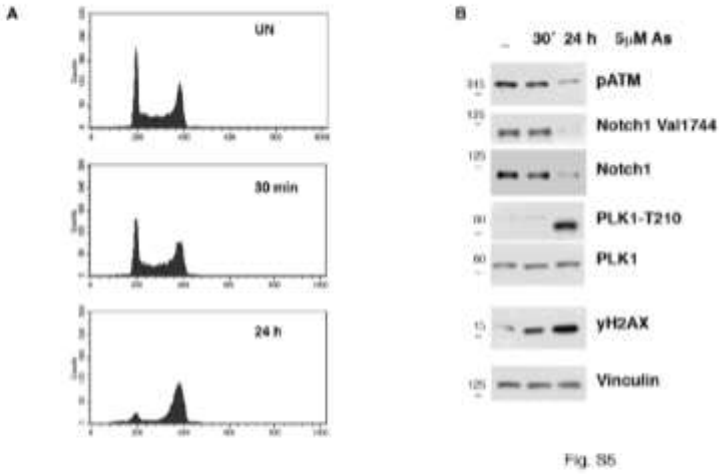

Fig. S5

Figure S5. PLK1 and NOTCH1 expression are inversely correlated in in As_2O_3-treated cells. **A)** HaCaT cells were treated for 30 min or 24 hr with Arsenite, then cells were collected and cell cycle analyzed by FACS. **B)** HaCaT cells were treated with As_2O_3 for 30 min or 24 hrs and analyzed by immunoblot with the indicated antibodies.

S6

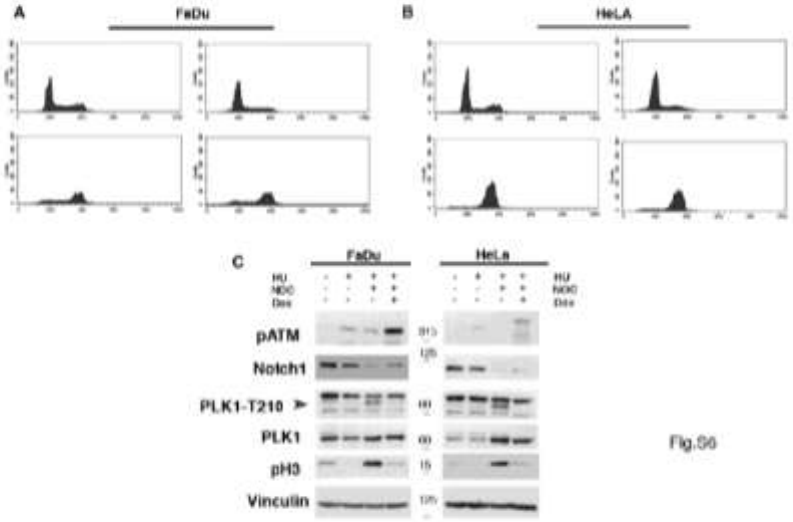

Fig.S6

Figure S6. NOTCH1 expression in G2 DNA Damage Arrest. **A,B,)** FaDu and HeLa cells were left untreated (diagram 1) or treated with Hydroxyurea for 19 hrs (diagram 2). Alternatively, cells were released from the HU block and either untreated or treated after 7 hrs with Doxorubicin for 1 hr and subsequently grown in the presence of Nocodazole for 18 hrs. Following these treatments, cells were collected at the indicated time-points after release from G1/S, cell cycle analyzed by FACS. **C)** FaDu and HeLa cells were treated as described in panel A, B and analyzed by western blot with antibodies against the indicated proteins. Shown are the representative results from at least 3 independent experiments.

IV. CONCLUSIONS

Hailey-Hailey disease (HHD) is a rare autosomal dominant skin disorder manifesting in the 3rd to 4th decades of life. The genetics and pathophysiology of this skin disorder have been linked to mutations in the *ATP2C1* gene. The gene, located on the long arm of chromosome 3, encodes the human secretory pathway Ca^{2+}/Mn^{2+} ATPase, hSPCA. The loss of ATP2C1 in keratinocytes leads to the loss of cell-to-cell adhesion (acantholysis) among the cells of the suprabasal layers (Sudbrak R. et al., 2000; Hu Z. et al., 2000; Wilgram G., Caulfield J. and Lever W., 1962). Moreover, even if rare, squamous/basal cell carcinomas and melanomas arising in the skin lesions have been described (Chun S. et al., 1988; Cockayne S.E. et al., 2000; Holst V. et al., 2000; Mohr M. et al., 2011).

There is no treatment known to be effective in reducing the cutaneous manifestations of HHD. The Standard of Care (SOC) treatment consists in either topical or oral administration of corticosteroids often used in combination with topical/systemic antimicrobial agents. However, prolonged treatment course of steroids is limited due to their side effects, most commonly skin atrophy. This last aspect must be carefully considered because in HHD-patients lesion development is associated with the simply friction of the skin and we found that HHD-keratinocytes are characterized by wound defect. Additionally, patients develop lesions refractory to corticosteroids.

In this line, the discovery of new therapies aimed to target the pathogenic mechanism underlying the disease is undoubtedly an important goal, in order to provide better and more efficient treatment conditions for HHD patients.

Unfortunately, researching on rare diseases such as Hailey-Hailey disease can be challenging primarily due to the limited number of individuals selected to participate in any given study and also due to ethical and psychological issues that must be carefully considered. In addition, mouse models poorly recapitulate the main human pathological features of HHD (Okunade G. et al., 2007).

In our works, we have explored the use of the yeast *K. lactis* as a tool to identify specific compounds that target specific cellular phenotypes and obtain more insight into mechanisms of disease pathology by probing the mechanisms

involved in their action. Oxidative stress represents a hallmark of both *Kluyveromyces lactis* lacking *PMR1* and keratinocytes derived from the lesioned areas of HHD patients and it could be associated with the decreased action of the transcription factor NRF2 involved in the regulation of several detoxifying factors. In addition to oxidative stress, HHD-derived keratinocytes are characterized by deregulated cytokines production and decreased repair properties.

In this context, we investigated the potential effects of compounds selected by a yeast-based screening assay, in an *in vitro* model of HHD. We found that treatment of ATP2C1-defective keratinocytes with the selected compounds, Kaempferol and hypochlorous acid, contributed to up-regulation of Nrf2 improving the cellular antioxidant response and the keratinocytes proliferation rate. Furthermore, we provided evidences of hypochlorous acid efficacy also on the pattern of cytokines produced. Notably, it was able to affect the deregulated cytokines expression by differentially modulate TGFB1 and TGFB2 levels.

Our results indicate that molecules able to counteract oxidative stress and to restore appropriate cytokines production and healing properties could be a viable therapeutic approach for HHD.

Malignant transformation is a multi-step process during which genomic instability and changes in the in the tissue microenvironment drive normal cells to a cancerous phenotype. Although HHD is a rare disorder and our knowledge is often related to few case reports, patients with squamous/basal cell carcinomas arising in the skin lesions have been described. HHD derived keratinocytes are characterized by an impaired DNA damage response which in turn is responsible for accumulation of mutations. Notch1 signaling plays a pivotal role in ATM and DDR repression in HHD keratinocytes. In order to get a better understanding of how Notch1 is involved in the DDR, we identified a novel mechanism through which Notch1 takes part in the genotoxic stress response. We further demonstrate that DNA damaged arrested cells employ the Notch1 signaling to protect them from damage-induced apoptosis and to release pro-inflammatory cytokines.

In summary, even though some aspects need to be more investigated, in the last years, our research activity has been extremely focused on the study of HHD contributing to increase the knowledge about its pathogenesis in particular on molecular level. Using several model systems, such as epithelial cells, primary human keratinocytes and yeast model, I have explored how loss of ATP2C1 function causes increased oxidative-stress, deregulation of Notch1 signaling and how alteration of Notch1 plays a crucial role in DNA damage response. Thus, our results indicate that an *ATP2C1/NOTCH1* axis might be critical for keratinocytes function and cutaneous homeostasis, suggesting a plausible model for the pathological features of Hailey-Hailey disease.

V. APPENDIX I

1. DNA Damage Stress: Cui Prodest? (Verma N., Franchitto M., Zonfrili A., et al., 2019).

International Journal of
Molecular Sciences

MDPI

Review

DNA Damage Stress: Cui Prodest?

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Received: 18 January 2019; Accepted: 26 February 2019; Published: 1 March 2019

Abstract: DNA is an entity shielded by mechanisms that maintain genomic stability and are essential for living cells; however, DNA is constantly subject to assaults from the environment throughout the cellular life span, making the genome susceptible to mutation and irreparable damage. Cells are prepared to mend such events through cell death as an extrema ratio to solve those threats from a multicellular perspective. However, in cells under various stress conditions, checkpoint mechanisms are activated to allow cells to have enough time to repair the damaged DNA. In yeast, entry into the cell cycle when damage is not completely repaired represents an adaptive mechanism to cope with stressful conditions. In multicellular organisms, entry into cell cycle with damaged DNA is strictly forbidden. However, in cancer development, individual cells undergo checkpoint adaptation, in which most cells die, but some survive acquiring advantageous mutations and selfishly evolve a conflictual behavior. In this review, we focus on how, in cancer development, cells rely on checkpoint adaptation to escape DNA stress and ultimately to cell death.

Keywords: cell cycle checkpoints; genomic instability; G2-arrest; cell death; repair of DNA damage; adaptation

1. Introduction

While questionable, one of the most well-known and widely reported aspect in cancer biology is the acquisition of genetic mutations that underlie cell transformation and tumor progression. From this perspective, cell transformation is a genetic process of tumor cells adapted to stressful environmental conditions; if to 'cell adaptation' can be conferred the Darwinian concept to respond to life's needs for survival, the nature of what adaptation means for tumor cells is extremely elusive. Either physical or chemical environmental agents can cause DNA damage and consequently genetic mutations that promote cell transformation.

Examples of physical agents promoting mutations are ionizing radiation, ultraviolet light present in sunlight which can promote the estimated rate of up to 10,000 DNA lesions per cell per day [1,2]; chemical agents such as benzo(a)pyrene B(a)P, 7,12-dimethylbenz[a]anthracene (DMBA), that generate DNA adducts, leading to mutations [3]. Beside exogenously, DNA damage can also occur endogenously as cells divide, with tens of thousands events every day in each single cell [2]. Thus, DNA damage might potentially affect the function of central regulators of many biological processes, ultimately leading to cancer development. Additionally, infectious pathogens elicit an oncogenic spiral that is one of the causes of cancer development [4]. If we assess the concept that 'adaptation' means the optimization of the phenotype whereby the organism acquires changes that increase its survival and reproductive success, when this concept is applied to cell transformation it remains extremely vague. Although this concept is suitable for viral carcinogenesis that hijacking cellular pathways

promotes the survival and proliferation of infected cells, in a multicellular organism, cells do not need to adapt their phenotype to a non-permissive environment. Unquestionably, in multicellular organisms, cells are immersed in growth conditions favorable to their replication. However, there is an obvious difference in the relationship between adaptation and environment in unicellular versus multicellular organisms. Life and replication in unicellular organisms are dependent on the conditions present in the environment and they survive if they are able to adapt to environmental changes. In sharp contrast, in multicellular organisms cell division is tightly regulated to control cell shape, tissue patterns, and morphogenesis [5], although cells are typically immersed in permissive environmental conditions. Preservation of the integrity of multicellular organisms relies on these extra layers of developmental control that function to restrain cellular proliferation that may change in response to environmental or intracellular stress signals. This implies that, as previously defined [6,7], cancer cells arise from cells adapted to respond to holistic control system and the escape from these host defense mechanisms represents an important strategy for cell transformation.

2. Cell Cycle Surveillance System

Genetic damage produced by either exogenous or endogenous mechanisms represents an ongoing threat to the cell. To preserve genome integrity, eukaryotic cells have evolved repair mechanisms specific for different types of DNA Damage (for an extensive review see [8,9]). However, regardless of the type of damage a sophisticated surveillance mechanism, called DNA damage checkpoint, detects and signals its presence to the DNA repair machinery. DNA damage checkpoint has been functionally conserved throughout eukaryotic evolution, with most of the relevant players in the checkpoint response highly conserved from yeast to human [10]. Checkpoints are induced to delay cell cycle progression and to allow cells to repair damaged DNA (Figure 1). Once the damaged DNA is repaired, the checkpoint machinery triggers signals that will resume cell cycle progression [11]. In cells, multiple pathways contribute to DNA repair, but independently of the specific pathway involved, three phase are traditionally identified: Sensing of damage, signal, and downstream effects (Figure 2). The sensor phase recognizes the damage and activates the signal transduction phase to select the appropriate repair pathway. For example, cells pose at least four independent mechanisms for repairing Double-Strand-Breaks (DSBs): Non-Homologous End-Joining (NHEJ), either classic-NHEJ or alternative-NHEJ, Homologous Recombination (HR), and single-strand annealing (SSA) [1,10,12,13]. Furthermore, highlighting the complexity of the DNA damage response, in mammals, at least four, in part, independent sensors can detect DSBs: Mre11-Rad50-Xrs2 (MRN), Poly ADP-Ribose polymerase (PARP), Ku70/Ku80 and Replication protein A (RPA) that binds single stranded DNA permitting the further processing of DSBs [1,14]. In the presence of DSBs, the activation of the DNA damage response and the mobilization of the repair proteins give rise to the formation of nuclear foci at the sites of damage. In yeast, the MRX-complex (Mre11-Rad50-Xrs2) is recruited at the site of DSBs [15]. Localization of MRX-complex to the damaged site is required to recruit and activate the protein kinase Tel1, which initiates DSBs signaling [13,16]. A similar mechanism is employed by MRN-complex in mammal cells (in which Nbs1 is the mammalian ortholog of Xrs2). MRN-complex orchestrates the cellular response to DBSs by physically interacting and activating the kinase Ataxia-Telangiectasia Mutated (ATM, the mammalian ortholog of Tel1). The signal is transduced by ATM that phosphorylates the histone variant Histone-2AX (H2AX) generating g-H2AX that promotes the recruitment of Mediator of DNA-Damage Checkpoin 1 (MDC1) protein at the site of damage. MDC1 amplifies the DNA-Damage Response (DDR) signal through the iterated recruitment of the MRN-ATM complex at the damage site that further phosphorylates adjacent H2AX molecules extending the γ-H2AX mark [13,16]. Additionally, MDC1 functions as an interaction platform for other DDR components including chromatin remodelers and ubiquitin ligase complexes [13,16]. The recruitment of these factors is essential to create a more open and accessible chromatin conformation to facilitate access at sites of DNA lesions and to allow ubiquitin-mediated accumulation of DNA repair factors, which will ultimately contribute to DNA repair pathways [13,16,17]. An integral part of the DNA damage

response is the parallel induction of repair mechanisms and reversible cell cycle arrest that delays cell cycle progression to give cells time for DNA repair [11]. The Checkpoint kinases 1 and 2 (CHK1 and CHK2) are key downstream effectors of DDR signaling as they promote cell cycle arrest. ATM/ATR phosphorylate and activate the CHK1 and/or CHK2 kinase [18]. While CHK1 and CHK2 have overlapping substrate preferences, they contribute differentially to the maintenance of the cell cycle checkpoint. A central mechanism in the induction of the checkpoint-induced cell cycle arrest is the inhibition of cyclin-dependent kinase(s) (Cdk). In this mechanism, ATM and CHK2 are required to both stabilize and increase p53 DNA binding activity which in turn results in the induction of its several transcriptional targets, among which the Cdk-inhibitor protein p21waf1/cip [19,20]. A central target involved in the activation of the cell cycle checkpoint mediated by both CHK1 and CHK2 is the Cdc25 family of phosphatases (Cdc25A, B and C) [9]. Cdks are in an inactive state when phosphorylated at two inhibitory sites, Thr 14 and Tyr 15. Removal of these phosphates by Cdc25 phosphatases results in the activation of CDKs and cell-cycle progression [9]. Thus, CHK1/2-mediated phosphorylation of Cdc25 proteins results in their functional inactivation, preventing CDKs dephosphorylation and activation [9,21]. Overall, in mammal cells, CHK1 is thought to be the primary effector of the G2/M phase checkpoints, whereas CHK1 and CHK2 exert a cooperative role in the intra-S and G1/S checkpoints [22].

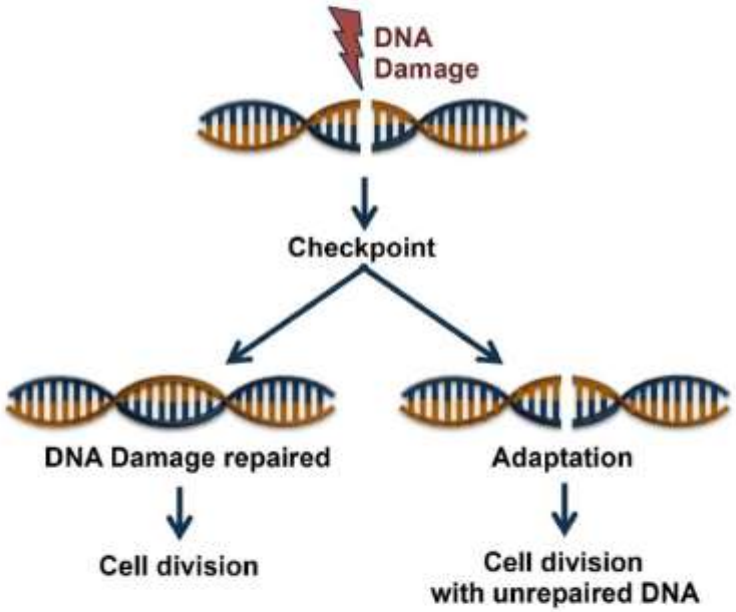

Figure 1. Cell fates following DNA Damage. Cell cycle checkpoint is induced by DNA damage. Cell cycle entry occurs after the DNA damages have been fully repaired, or alternatively, cells have two possible fates, to die or survive after a process of adaptation that allows cell division with unrepaired DNA lesions.

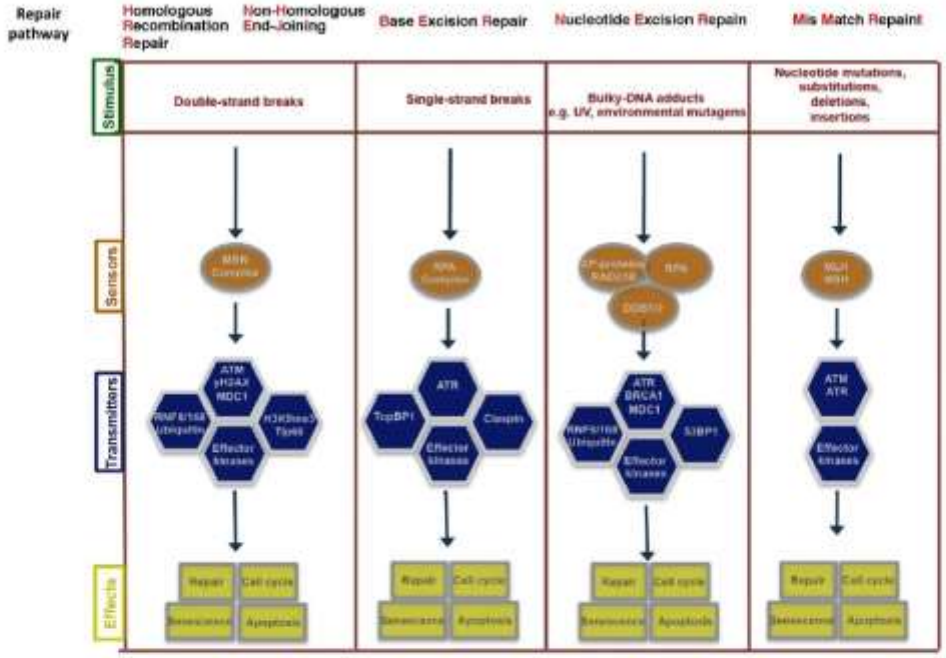

Figure 2. Schematic representation of the sensors, transducers and mediators involved in DNA damage response (DDR) pathways. DNA damage response is sensed and repaired by multi-protein complexes. Depending on the level of injury, the signaling triggered by the damage response will result in different cellular fates.

3. After Event Cleaning Job: RELEASE of the DNA Damage Checkpoint

The DNA Damage response elicits the activation of a highly complex and synchronized network of factors, such as kinases, phosphatases, transferases, and ligases [23–27]. Most of these enzymes add to remove functional groups that reversibly change the proteins fate or function [23–27]. Thus, when genome integrity is re-established the removal of these post-translational modifications is essential for a rapid checkpoint silencing and cell cycle progression [13]. Distinct DNA damage checkpoints at different stages of the cell cycle, such as G1/S, intra-S, and G2/M, have been described [28]. However, the exact dynamic and molecular basis of the recovery phase still remains not entirely clear. Recently, it has been shown that cell's response to DSBs depends on its cell cycle phase and that checkpoint dynamics are phase-dependent [28]. In the G1 phase, DBSs completely halt the cell cycle only in the presence of high DNA damage levels. The most abrupt and complete halt to the cell cycle occurs during G2/M, and interestingly, cell cycle arrest is linearly correlated with the amount of DNA damage [28]. The S phase checkpoint is the more permissive to DNA damage and allows cell cycle progression, although at a greatly reduced rate [28]. However, multiple layers of complexity exist in order to prevent cell cycle progression in the presence of damaged DNA. Cell cycle progression occurs in a linear manner, in which each checkpoint functions as an additional layer of control of the previous checkpoint. Thus, the G1 checkpoint is important in cells that have been exposed to DNA damage in the G1-phase, as well as for those that have been adapted from the G2 checkpoint [29]. In this context, it is interesting to note that, conversely to the redundancy of factors and mechanisms that share a temporal and overlapping function in response to DNA damage, checkpoint recovery relies on the involvement of phase-specific factors [13]. The CDC25B is a S/G2 phosphatase that is thought to play an essential role in activating CDK1-cyclin B complexes at the entry into mitosis ([13] and references there in). CDC25B has been shown to cooperate with the polo-like kinase 1 (PLK1) in promoting the cell cycle resumption in G2 phase after DNA damage. In addition, recovery of the

G2 DNA damage checkpoint appears to be distinct from G1. Indeed, both PLK1 and Cdc25B are not expressed in G1 and do not influence cell cycle resumption in G1 (Reference [13] and references therein). Essentially the same activation pathways promote mitotic entry in an unperturbed cell cycle and checkpoint recovery [30]. However, these pathways are thought to be differentially involved in these two processes. PLK1 is not essential for mitotic entry in cells progressing through normal cell cycles; it has been shown that the complete inhibition of PLK1 can only delay G2/M transition leaving the importance of PLK1 for mitotic entry during unperturbed cell cycle controversy [13,31]. Conversely, it is well established that initiation of the DNA damage response repress pro-mitotic machinery and leads to the inhibition of pro-mitotic kinases among which CDK1, Aurora A, and PLK1 [32–34]. Additionally, the degradation of Cdc25 and Bora, as well as of several other proteins involved in mitotic entry, is critical for cell cycle arrest [35,36]. While PLK1 is dispensable for the onset of mitosis in an unperturbed cell cycle, in sharp contrast PLK1, is essential for mitotic entry following recovery from DNA Damage-induced cell cycle arrest [37]. Cell cycle re-entry relies on the Aurora-A kinase and its co-factor Bora, which phosphorylates PLK1 at Thr210 in its activation loop; thus, Plk1 is activated and promotes mitotic entry by stimulating cyclin B1-Cdk1 activation [25,30,37,38]. PLK1 can promote cyclinB1/CDK1 activation by several mechanisms. Early works in *Xenopus* have established that Plx1 (PLK1) phosphorylates and activates Cdc25C, and this activates the Cyclin B–CDK1 complex. In vertebrates, the Cdc25 paralogues (Cdc25A, B and C), all have been shown to be target of PLK1 activity [39], but it remains poorly characterized, with Cdc25 phosphatase(s) the substrate of PLK1 during the G2 recovery. However, it has been suggested that G2 recovery is dependent on the specific isoform Cdc25B, which is stabilized after damage, while Cdc25A expression is reduced [37,40]. Beside its implication in the re-activation of cyclin-B1–CDK1 complex, PLK1 controls the silencing of DDR signals by inactivating the ATM/CHK2 pathway. Within the DNA damage response mechanism, 53BP1 is an adaptor protein required to tether several checkpoint components at the damaged sites, including CHK2 and ATM. In PLK1-mediated inactivation of the DNA damage checkpoint, it has been shown that PLK1 phosphorylated 53BP1 that thus fails to form foci after DNA damage [41]. Additionally, it has been shown that PLK1 also directly phosphorylates and inactivates CHK2 [41]. Thus, PLK1 negatively regulates the ATM-CHK2 branch of the DNA damage to inactivate checkpoint signaling and to control checkpoint duration [41]. Similarly, PLK1 negatively controls Claspin and CHK1 and the inactivation of these components results in a shutdown of the checkpoint [42–44]. Specifically, phosphorylation of Claspin by PLK1 creates a docking site for β-TrCP protein, resulting in the efficient ubiquitin-mediated degradation of this protein [42–44]. In conclusion, PLK1 is capable of driving entry into mitosis after DNA damage-induced cell cycle arrest and to promote checkpoint silencing and recovery.

4. DNA Damage and the Balance between Survival and Death

A central question in cells responding to DNA damage is how DDR pathway controls cell fate decision. The accepted paradigm implies that the level of damage may trigger different responses; thus, low-level promotes the initiation of repair and the activation of survival mechanisms, whereas high-levels promote cell death. This concept includes the tacit assumption that, if the damage is irreparable, cells undergo apoptosis; however, there currently is not a clear biochemical mechanism for how cells distinguish between reparable and irreparable DNA damage. Evidence suggests that cells respond to DNA damage by simultaneously activating DNA repair and cell death pathways [45,46]; p53 protein and its functional ambiguity might play a central role in this context, given the ability of p53 to control the transcription of genes involved in either survival or death [47]. p53 influences several pathways, which are essential for progression through the cell cycle, including G_1/S, G_2/M and spindle assembly checkpoints [48]. Thus, it is not surprising that several signaling pathways can converge on p53 to control cellular outcomes. Among them, PLK1 was shown to physically bind to p53 inhibiting its transactivation activity, as well as its pro-apoptotic function [49]. As mentioned above, upon DNA damage, ATM/ATR alone lead to phosphorylation of several hundreds of proteins, among them

p53 [50]. The Mouse Double Minute 2 protein (MDM2) represents one of the predominant and critical E3 ubiquitin ligase for p53, responsible for the dynamic regulation of p53 function [51–54]. MDM2 mediates p53 ubiquitination through a RING domain (Really Interesting New Gene domain). Additionally, p53 and MDM2 function in a negative feedback loop, in which MDM2 transcription is activated by p53 and under normal stress conditions, MDM2 maintains low levels of p53 protein [51–54]. Furthermore, it has been observed that MDM2 binds to the promoters of p53-responsive genes and form a complex with p53 by interacting with its transactivation domain, thus MDM2 mediates histone ubiquitylation and transcriptional repression of p53 targets genes [51–54]. Upon DNA damage, ATM/ATR either directly or through CHK1/CHK2 phosphorylate p53 (Reference [46] and references there in). Similarly, it has been shown that ATM phosphorylates MDM2 (References [46,55] and references therein); phosphorylation of p53 and MDM2 in response to DNA damage by ATM/CHK1/CHK2 is thought to abrogate the MDM2-p53 protein-protein interaction leading to p53 stabilization and activation. (References [46,55] and references therein). In this context, it is thought that a low-level of DNA damage causes a transiently expression and response of p53 whereas a higher-level of DNA damage leads to sustained p53 activation. Thus, upon DNA damage cell fate is determined by tunable threshold of p53. Previous studies have indicated that p53 may selectively contribute to the differential expression of pro-survival and pro-apoptotic genes, due to the higher affinity of p53 for its binding sites in promoter associated with cell cycle arrest, e.g p21/CDKN1A and lower affinity for those associated with apoptosis [47]. It has been shown that both pro-arrest and pro-apoptotic p53 target genes are expressed proportionally to the p53 expression levels [47]. It is conceivable that, upon DNA damage triggering apoptosis, cells must reach the pro-apoptotic threshold of p53 activity, whose level is determined by expression levels of p53 itself. Interestingly, it has been shown that lowering this pro-apoptotic threshold with inhibitors of antiapoptotic Bcl-2 family proteins sensitized cells to p53-induced apoptosis [47]. DNA damage can activate both p53 and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). A model describing the crosstalk between p53 and NF-κB was proposed by Puszynski and co-workers [56]. This work suggested that the diverse outcome of the p53/NF-κB crosstalk in balancing survival and death depended on the dynamic context of p53 and NF-κB pathways activation. It has been proposed that NF-κB activation preceding p53 activation render cells more resistant to DNA damage-related death [56]. Remarkably, data from gain and loss of function approaches demonstrated that sustained anti-apoptotic NF-κB activity in tumors might depend on mutant p53 activity [57]. Thus, the regulation of p53 and its downstream effects are likely to be dependent on its interaction with other signal transduction pathways, which may influence the final response to p53 activation. In addition to the above-discussed mechanisms that control p53's duality in cell fate, site-specific phosphorylation of p53 also seems to be important in promoting its pro-apoptotic function. It has been observed that promoter selectivity of p53 is regulated by post-translational modifications [58]. In this context, the increased affinity of p53 to the regulatory regions of pro-apoptotic genes is related to its phosphorylation at serine-46 (ser46) [58]. Thus, in stress-conditions, phosphorylation of p53 at S-46 regulates its pro-death function through the induction of apoptotic genes such as *NOXA* [59] *PTEN* [60] and *TP53AIP1* [61]. Several kinases phosphorylate p53 on S-46 either directly (HIPK2, p38, PKCδ, and DYRK2) or indirectly through ATM/ATR, with the effect to promote upregulation of pro-apoptotic p53-target genes [62–66]. In addition to its role as regulator of the cell fate of genomically compromised cells, several studies have shown that p53 also directly impacts the activity of various DNA-repair pathways [67]. Thus, p53 appears a multitasking factor providing protection from cancer development by maintaining genome stability. In conclusion, p53 is a central component of the signaling network activated by the DNA damage response and the tight regulation and balance of its activity must be maintained to preserve the dynamic principle of the damage checkpoint.

5. Molecular Mechanisms of Checkpoint Adaptation

Cells have evolved a complex network to maintain the integrity of the genome. An essential event in the DNA damage response is represented by the cell cycle arrest that allows cells to repair damaged

DNA before entering the subsequent phases of the cell cycle [11]. Thus, the expected consequence in the presence of DNA damage is that cell cycle re-entry will only occur following DNA repair [11]. However, cells can enter into cell cycle before repairing their DNA through a mechanism originally described as checkpoint adaptation [68–70]. While in mammal cells the molecular mechanism of checkpoint adaptation has remained controversial and largely unknown until recently, it has been extensively studied in *Xenopus* and yeast. Since the checkpoint adaptation and checkpoint recovery mechanism share keys factors, it is not surprising that components of the checkpoint adaptation response are highly conserved throughout the eukaryotic evolution [10]. In the yeast *S. cerevisiae*, analysis of deletion mutants indicates that multiple factors are involved in checkpoint adaptation, among them: Cdc5 (PLK1), Tel1 (ATM), and Mec1 (ATR) [16]. In response to different kinds of DNA damage, checkpoint activation promotes the recruitment of Tel1/Mec1 to the lesion site [15]. The Tel1/Mec1 kinases directly phosphorylate the adaptor proteins Rad9 and Mrc1 that are able to recruit and to activate the checkpoint Kinase Rad53, the structural homolog of human CHK2, but considered functionally similar to CHK1 [71]. Phosphorylation of Rad53 as well as that of CHK1 promotes cell cycle arrest [15,71–73]. Several observations indicate that inhibition of Rad53 plays a crucial role in the control of the adaptation process; in particular, Rad53 over-activation was observed in diverse adaptation-defective mutants [73]. Moreover, it has been shown that Cdc5-mediated phosphorylation of Rad53 is required for checkpoint adaptation [74]; consistently with the finding that a dominant negative Rad53 mutant was shown to bypass the requirement of cdc5, in a cdc5 adaptation-defective mutant [73]. Finally, Rad53 de-phosphorylation mediated by both the phosphatases Ptc2 and Ptc3 has been shown to bypass the DNA damage checkpoint [65,72,75]. Thus, most of the common pathways involved in checkpoint adaptation inhibit Rad53 to promote entry into the cell cycle.

A consistent link between the Plx1 (PLK1) and Chk1 has been also observed in *Xenopus laevis* [76]. Persistent replication stress promotes the interaction between Claspin and Plx1, which causes the phosphorylation and release of Claspin from the chromatin and thereby Chk1 inactivation [76]. While checkpoint adaptation has been extensively studied in both lower and higher eukaryotes, its existence in mammal cells has long been considered controversial [10,77]. However, soon after the studies cited above, several authors reported a similar type of functional interaction between PLK1 and CHK1 in human cells. Overall these studies depict a model in which PLK1 phosphorylates and promotes $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase-mediated processing of Claspin, thereby promoting CHK1 de-phosphorylation and inactivation [43,44,78]. Based on these studies, *PLK1* has attracted a lot of interest for understanding the molecular mechanism controlling checkpoint adaptation. Thus, a number of experimental observations have provided mechanistic insight into the involvement of PLK1 in checkpoint adaptation. Interestingly, was observed that in the presence of DNA damage PLK1 degradation is required to achieve a proper G2 arrest [79], consistently with previous observations indicating that sustained PLK1 activity following DNA damage increases the fraction of mitotic cells [33]. In addition to Claspin, it was shown that in checkpoint adaptation WEE1 kinase is a direct downstream target of PLK1 (Reference [37] and references there in) WEE1 negatively regulates entry into mitosis by promoting the phosphorylation of CDK1, thus inhibiting the CDK1/cyclin B complex. PLK1 phosphorylates and leads to degradation WEE1, thereby promoting entry into mitosis [Reference 37 and references therein]. The requirement of PLK1 activity in cells entering in mitosis it has been elegantly confirmed by using a fluorescence-based probe for PLK1 activity at single cell level [80]. It has been reported that increased PLK1 activity is detected in cells entering mitosis in unperturbed cell cycle and when cells recover from DNA damage checkpoint by addition of caffeine that force a shutdown of the checkpoint [25,80,81]. An interesting observation arising from these studies is that, once PLK1 activity increases beyond a certain level, it overrides damage checkpoint regardless of whether DNA damage persists [80].

However, while a number of studies favor the notion of a central role of PLK1 to drive checkpoint adaptation, likely there are multiple factors that contribute to the DNA damage recovery. CDK1 is a key regulator of mitotic entry, and as discussed above, PLK1 itself can phosphorylate it. Thus, it is

likely that signaling pathways able to influence Cyclin B/CDK1 activity in conjunction with PLK1 potentially might regulate adaptation [13,16,37].

6. Consequences of Checkpoint Adaptation

Cell cycle checkpoints and DNA repair mechanisms are important processes to maintain the integrity of the genome and the faithful transfer of genetic information to daughter cells [10]. This surveillance mechanism provides time to repair the damage, and only when repair has been successful, the checkpoint is extinguished and cells re-enter into the cell cycle [1,10,12,46,77,82,83]. In unicellular organisms, if DNA repair is not possible, cells can overcome DNA Damage through checkpoint adaptation [15,21,71,77,84]. Interestingly, mounting evidence indicates that this concept is not only found in unicellular eukaryotes like yeast but it might be extended also in multicellular organisms [10,16,76,77,85]. While the critical determinants of the outcomes of checkpoint adaptation are not yet precisely understood, checkpoint adaptation has several possible consequences. For instance most cells that undergo checkpoint adaptation die, whereas some cells survive; surviving cells face two different fates: Some cells will die in subsequent phases of the cell cycle, but a small number of cells will survive and divide with damaged DNA [References [85–87] and references there in]. In line with this model, it has been demonstrated that in repair-defective diploid yeast, nearly all cells undergo checkpoint adaptation, resulting in the generation of aneuploid cells with whole chromosome losses that have acquired resistance to the initial genotoxic challenge [84]. An important consequence of this finding was the demonstration that adaptation inhibition, either pharmacologically or genetically, drastically reduces the occurrence of resistant cells [87–89]. Thus, both in unicellular and multicellular organisms checkpoint adaptation might represent a mechanism that increases cells survival and increases the risk of propagation of damaged DNA to daughter cells [86,87,89]. Understanding this aspect is particularly important as a weakened checkpoint, it has been shown, enhances both spontaneous and carcinogen-mediated tumorigenesis [90,91]. Additionally, DNA damaging agents are widely used in oncology to treat many forms of cancer [92]. Unfortunately, resistance to these agents can result from a variety of factors that significantly reduce their efficacy in cancer therapy [93]. There is evidence that checkpoint adaptation may drive the selection of therapy-resistant cells (Reference [92] and references therein). A better understanding of the mechanisms that determine either survival or death following checkpoint adaptation might provide insight into the potential mechanisms for the failure of cancer therapies, thereby facilitating further improvement of current cancer treatments.

7. Future Directions

Cancer is often regarded as an asexual evolution in which cancer cells arise through the sequential acquisition of beneficial mutations that should confer an increased fitness to the adapted cells [94–96]. Checkpoint adaptation serves as a mechanism by which cells become adapted to stressful conditions [16,77,84,85,89,92]. As described above, in this process the interaction between DNA repair pathways and cell cycle checkpoints determines cell fate decision and prevents neoplastic transformation. Preservation of integrity of multicellular organisms relies on these extra layers of developmental control. While the nature of what adaptation means for tumor cells in a multicellular organism remains puzzling, several observations indicate that the DNA Damage response may also affect the biology of the surrounding cellular microenvironment (for review see Reference [97]). In this process, the DNA damage response in cancer cells produces a paracrine signaling to induce changes in nearby microenvironment. However, DNA-damage response plays a crucial role, not only in cancers, but also in a wide variety of hereditary as well as non-genetic diseases [98–102]. A better understanding of how the DDR-driven signals are regulated and received by the surrounding microenvironment could represent an opportunity to understand how the systemic homeostasis controls cell fitness.

Funding: This resaerch was funded by the Associazione Italiana per la Ricerca sul Cancro, AIRC and by the Italian Ministry of Education, University and Research—Dipartimenti di Eccellenza—L. 232/2016. The APC was funded by Associazione Malati di Hailey-Hailey Disease, A.AMA.HHD-Onlus.

Conflicts of Interest: The authors declare no conflict of interest.

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