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"Analysis of the role of the secretory pathway Ca^{2+}/Mn^{2+}- ATPase, PMR1/ATP2C1, in calcium homeostasis: from oxidative stress to skin disease."

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SUMMARY

In this work, the genetically tractable *Kluyveromyces lactis* yeast has been used to study the molecular basis of Hailey–Hailey disease (HHD). This pathology, also known as familial benign chronic pemphigus, is a rare, chronic and recurrent blistering disorder, characterized by acantholysis. The genetics and pathophysiology of HHD have been linked to mutations in ATP2C1. The gene encodes for an adenosine triphosphate (ATP)-powered calcium channel pump and *PMR1* is the yeast orthologue. Similarly to human HHD-cells, in yeast the loss of *PMR1* promotes cellular toxicity caused by increased oxidative stress linked to the alteration of calcium homeostasis. By a functional suppression screening of yeast mutant with a cDNA library, we found that Glutathione S-transferase (GST), an important detoxifying enzyme, could be a candidate gene associated with Hailey-Hailey disease. Expression of mammalian GST in *Klpmr1Δ* alleviated several defects; these include oxidative-stress toxicity, calcium alteration and mitochondrial dysfunction. Additionally, the discoveries made in yeast were validated in HHD-derived cells. Indeed, HHD-lesional derived keratinocytes showed a decreased expression of GST gene when compared to non-lesional skin derived cells from the same patients. In parallel to the genetic screening, a drug screen was performed to find compounds with potential therapeutic effects for HHD treatment. Indeed, currently, there is no reported cure for HHD. For this reason, 131 FDA-approved natural compounds were screened for their ability to alleviate the calcium alterations and the oxidative stress of *Kluyveromyces lactis pmr1Δ* cells. The drug collection includes inhibitors, activators and antagonists acting on molecular targets involved in different signaling pathways. The initial screening system was based on the cell wall alterations of the *pmr1*Δ mutant, utilizing the chitin binding stilbene fluorescent brightener calcofluor white. In the first part of the screening 12 molecules resulted toxic, while 71% of the library molecules were ineffective and the remaining appeared to have a partial effect. Therefore, in a second round of the screening, these last compounds were evaluated at higher concentrations. Based on this, six molecules were selected for further analysis. Their effects on other defects of *pmr1Δ* mutant strain were

also evaluated, ranging from alteration of Ca^{2+} homeostasis to sensitivity to the ROS-generators menadione and H_2O_2, as well as the mitochondrial functionality.

INTRODUCTION

1. Calcium homeostasis

1.1. Calcium homeostasis in high eukaryotes

Ca^{2+} ions have a central role in cell signaling as a second messenger, regulating several cellular processes such as metabolism, cell proliferation, division and differentiation, gene transcription, muscle excitation-contraction, programmed cell death and neurotransmission (Berridge et al., 2003). To maintain Ca^{2+} homeostasis, the flow of Ca^{2+} into and out of cells and organelles has to be precisely regulated. A network of Ca^{2+}-transporters, -channels, -exchangers, -binding/buffering proteins and -pumps are involved to control within the cell, the fluxes and concentration of Ca^{2+} (Krebs et al., 2015).

1.1.1. Calcium buffers

Once Ca^{2+} enters in the cell, it is rapidly buffered by Ca^{2+} buffering proteins and the amount that escapes will then activate the targets of the signalling. In the cytosol there are the pure Ca^{2+} buffers (parvalbumin, calbindins, and calreticulin) and the Ca^{2+}sensors that not only buffer calcium but also process its signal. The most important of them is calmodulin (CaM), which is expressed ubiquitously in cells (Faas et al., 2011). It belongs to the family of EF-hand proteins and when it binds the Ca^{2+}, it undergoes under conformational changes to contact the target proteins (Kawasaki et al., 1998).
Also in the lumen of the intracellular stores, large amount of Ca^{2+} is buffered by Ca^{2+} binding proteins such as calsequestrin or calreticulin (Marenholz et al., 2004).

1.1.2. Calcium entry

Several plasma membrane Ca^{2+} channels allow the calcium enter in the cell (Fig. 1). These channels have been divided in three major groups: the voltage-gated channels (VOCs), the receptor-operated channels (ROCs), the store-operated Ca^{2+} entry channels (SOCEs).
The VOCs are key transducers of membrane potential localized in the conductive cells. Their Ca^{2+} selectivity is high and they can be divided in three subfamilies: Cav1, Cav2, and Cav3. The Cav1 subfamily initiates muscle contraction, secretion, regulation of gene expression, integration of synaptic signals, and mediates the L-type current. Cav2 subunits are mainly responsible for the initiation of synaptic transmission at fast synapses. The Cav3 subfamily is important for the action potentials in cardiac myocytes and thalamic neurons (Catterall, 2011).

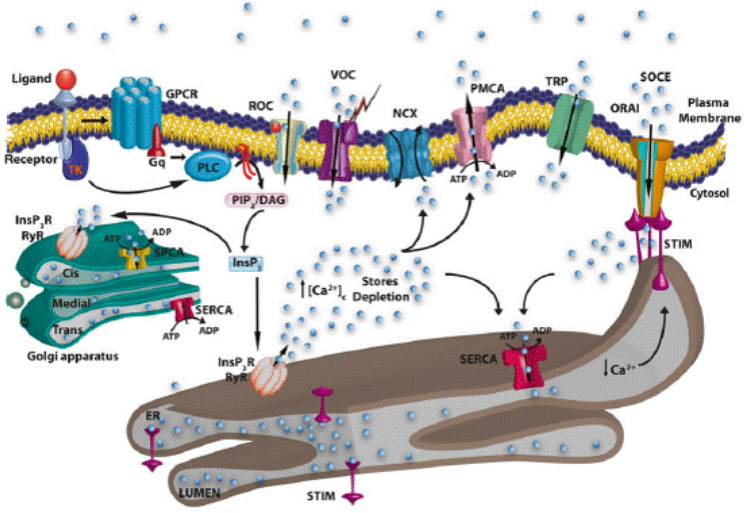

Fig. 1 Schematic representation of the different Ca^{2+} transport proteins of the plasma membrane and the main intracellular stores (Brini et al., 2013).

The second class of membrane Ca^{2+} channels, ROCs, is activated by the interaction with ligands. For example, L-glutamate, the most important excitatory transmitter in mammalian brain, activates two classes of receptors, the ionotropic receptors (iGluRs) and the metabotropic receptors (mGluRs). The first are ligand-gated non-selective cation channels, while the second are coupled to G-proteins and they generate Ca^{2+} signals through the activation of distinct downstream signaling cascades.
The SOCEs are instead activated by the release of Ca^{2+} from the endoplasmic reticulum (ER). They are composed by two elements: a Ca^{2+}-binding transmembrane protein, STIM (stromal interacting molecule), that serves as sensor of calcium within the ER and a plasma membrane store-operated channel composed by ORAI subunits(Gwack et al., 2007).
When there is a depletion of calcium from ER, these two protein localized to the ER-PM junctions, coupled together to form the SOCE channel, thus generating a localized Ca^{2+} influx (Luik,et al., 2006).
TRPCs (transient receptor potential channels) instead contribute to change the intracellular Ca^{2+} concentration either directly by acting as a calcium entry pathway, or indirectly by changing the membrane polarization that in turn may trigger the activation of different voltage-dependent ion channels (Montell et al., **2005**; Gees et al., **2010**).

1.1.3. Intracellular calcium release

The intracellular Ca^{2+} Channels, are localized on the endo/sarcoplasmic reticulum (ER/SR) and the Golgi apparatus (GA) (Fig 1). The main two types of Ca^{2+} receptors/channels in these compartments are the ubiquitous inositol 1,4,5-trisphosphate receptors (InsP3 R) and the ryanodine receptor (RyR), which is not present in all cell types (Chan et al., 2010). When the cell surface receptors are activated, the second messenger InsP3 is generated through the activity of phospholipase C and thus it releases calcium from the endoplasmic reticulum through the InsP3R (Taylor and Tovey, 2010). Cytosolic Ca^{2+} is a co-agonist of the InsP3Rs, so that it strongly increases its activity at concentrations

up to about 300nM. By contrast, at higher concentrations it inhibits the receptor. Homologous to the IP3R is the Ca^{2+}-release channel of the sarcoplasmic reticulum, the ryanodine receptor (RyR), which received its name due to the high affinity binding of the plant alkaloid ryanodine (Petegem, 2012). In muscle cells, the RyR is primarily located on the SR, in non-muscle cells it is expressed in the ER. This receptor is responsible for the excitation-contraction mechanism-mediated coupled with the voltage-dependent Ca^{2+} channel dihydropyridine receptor (DHPR) located in the T-tubules of muscle cells. (Brini et al., 2013).

1.1.4. Calcium pumps

Ca^{2+}-ATPases (pumps) are another class of protein that contribute to regulate calcium levels in the animal cells. They are divided in to three classes: the plasma membrane Ca^{2+}-ATPase (PMCAs), the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCAs), and the secretory pathway Ca^{2+}-ATPase (SPCA) that are in the Golgi membrane. Meanwhile, in the SERCA pumps there are two Ca^{2+} binding sites (Clarke et al., 1990), the PMCA and SPCA pumps have only one, corresponding to site 2 of the SERCA pumps (Guerini et al., 2000).
They decrease the concentration of cytosolic Ca^{2+} by exporting it to the external medium, or to the internal space of the vesicles of the reticulum and of the Golgi systems. The three pumps have a high affinity for calcium and belong to the superfamily of P-type ATPases (Pedersen and Carafoli, 1987), which conserve temporarily the energy liberated by the splitting of ATP in the form of an aspartyl phosphate in their reaction center. The affinity for calcium is particularly high in the SPCA pumps, which have K_{ds} well below the concentration of Ca^{2+} in the cytosol at rest. This ensures that the Golgi vesicles will be always filled with calcium even in the absence of a calcium signalling activation. This is crucial, since Ca^{2+} is required for the activity of enzymes within the Golgi vesicles, like the endoproteases that process the pro-hormones. Importantly, the SPCAs also transport Mn^{2+}, which

is essential inside the Golgi vesicles for the O- and N-glycosylations of a number of proteins (Kaufman et al.,1994; Varki, 1998).
The plasma membrane possesses also Na^+/Ca^{2+} exchangers (NCXs) with a low Ca^{2+} affinity. The system belongs to the SLC8 (solute carrier family 8) that are particularly active in the cells of excitable tissues. NCX uses the energy of the electrochemical Na^+ gradient to export Ca^{2+}, with a transport stoichiometry of 3 Na^+ for 1 Ca^{2+} (Carafoli et al., 2001). Since the operation of the NCX is electrogenic and voltage-sensitive, it can reverse during cell activation and lead to the uptake of Ca^{2+}into the cell (Blaustein and Lederer, 1999). Another member of the Na^+/Ca^{2+} exchanger superfamily is the NCKX, that also exchanges K^+ and it was first identified in the retina (Reilander et al., 1992).

1.1.5. Other calcium transports

As another important calcium store also mitochondrion possesses different calcium transporters (Fig. 2). Calcium mainly enters in this organelle by Ca^{2+} uniporter MCU that uptakes Ca^{2+} released from ER (De Stefani et al., 2011). Meanwhile, in the case of Ca^{2+} efflux, mitochondrial Na^{2+}/ Ca^{2+} exchangers (NCLX) use the concentration gradient of Na^+ across the inner membrane to cause the release of Ca^{2+} back into the cytosol (Carafoli et al., 1974). The cycle is then completed thanks to the efflux of Na^+ via the Na^+/H^+ exchanger (NHE) (Nicholls, 2005).
Finally, acidic organelles, such as the acidic endosomes and lysosomes seem to be possible Ca^{2+} stores in mammalian cells (Fig. 2). Indeed, they possess Ca^{2+} - binding proteins, such as chromogranins and secretogranins, with a large Ca^{2+} - buffering capacity (Yoo et al., 2007) and they have also been reported to be able to release Ca^{2+} by canonical second messengers described for the ER/SR and the Golgi, like InsP3 and ryanodine. The calcium uptake through this system appears to rely on the large proton gradient established by the vacuolar proton pump V-ATPase (Patel and Docampo, 2010).

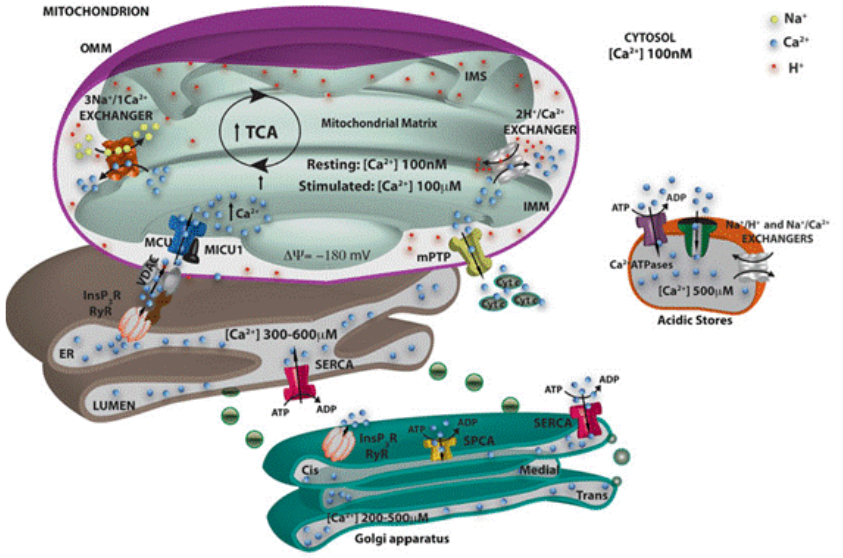

Fig. 2 Schematic representation of Ca^{2+} handling by mitochondria, endoplasmic reticulum (ER), Golgi apparatus, and acidic stores (Brini et al., 2013).

1.2. Calcium regulation in yeast

As in mammalian cells, calcium signalling regulates in yeast a wide variety of cellular processes including cell-cycle progression, mating, protein processing, responses to hypotonic stress, maintenance of intracellular pH, nutritional and metabolic signalling.
Furthermore, yeast cells also bear homologs of the major components of the mammalian Ca^{2+} signalling, including channels, co-transporters and pumps. Thus, it has proven to be a powerful tool to elucidate the molecular aspects of several Ca^{2+}-related processes of higher eukaryotes (D'hooge et al., 2015).

1.2.1. Calcium influx

Extracellular calcium can enter the cytosol through different pathways (Fig.3). The best characterized is the high affinity, low capacity influx system, homologue of voltage-gated Ca^{2+} channels. It is based on two Mid1p and Cch1p proteins that assemble on plasma membrane to form a channel (Paidhungat and Garrett 1997), but evidence also suggests that they may function independently (Locke et al. 2000). Both assembled on plasma membrane, Cch1 (Calcium Channel Homologue) was primarily identified as a Ca^{2+} channel homologue of a voltage-gated calcium channel from higher eukaryotes (Paidhungat and Garrett 1997); while Mid1 (Mating pheromone-Induced Death) was identified in a screen for mutants defective in survival after mating differentiation and in calcium uptake (Iida et al. 1994). Later, Mid1 was classified as a stretch-activated channel, with similarities to higher eukaryotes SA-Cat channels (Kanzaki et al. 1999). Mid1 was also found in ER as a 200-kDa oligomer by covalent cystein bounding (Yoshimura et al. 2004). Importantly, *mid1cch1* double mutants are indistinguishable of single mutants, this and physiological data indicating that Mid1 and Cch1 might be components of a unique yeast Ca^{2+} channel (Paidhungat and Garrett 1997). Both proteins together could act as a voltage-gated Ca^{2+} channel (VGCC) becoming activated in response to depolarization (Catterall 2000; Cui et al. 2009a). The presence of mating pheromone

or depletion of manganese from the medium (Paidhungat and Garrett 1997), depletion of calcium from the ER (Bonilla et al. 2002), alkaline environment (Viladevall et al. 2004), osmotic or saline stresses (Matsumoto et al. 2002; Peiter et al.2005; Viladevall et al. 2004), are signals that trigger calcium entry in the cell through the Mid1/Cch1 channel.
Based on calcium tolerance experiments and mathematical models, two transport systems have been postulated (Cui and Kaandorp 2006). These transporters or pumps have been termed as transporter X and transporter M and their activities could be modulated by extracellular Mg^{2+}.

1.2.2. Calcium storage

Excessive free calcium is toxic for yeast cells because it may interact with numerous proteins or oligomolecules (ie. polyphosphate-derived compounds such as NTPs) in the cytoplasm. Thus, the excess of calcium is rapidly eliminated by the activity of different Ca^{2+} pumps and exchangers (Cui et al. 2009b).
Vacuole is the main calcium store in the yeast and it contains approximately ≥95% of total cellular Ca^{2+} (Cui et al. 2009a). Calcium enter in the vacuole mainly through two principal transporters: the P-type ATPase Pmc1 pump (Cunningham and Fink 1994) and the Ca^{2+}/N^{+} exchanger, Vcx1 (Miseta et al. 1999).
Pmc1 is conserved in nearly all fungi and is closely related to the PMCA-family of plasma membrane Ca^{2+}-ATPases found in animals. However, Pmc1 has not been observed in the plasma membrane of *S. cerevisiae* but only in the vacuole membrane (Marchi et al., 1999).
When intracellular calcium levels increased, the expression of *PMC1* is up-regulated (Marchi et al. 1999) and loss of Pmc1 function reduces the amount of non-exchangeable calcium in the vacuole. However, inactivation of calcineurin restores calcium sequestration in the vacuole of a null *pmc1* mutant, indicating that tolerance of null *PMC1* mutants to calcium is dependent on the activity of Vcx1, which, on the other

hand, it is dependent on calcineurin activity (Cunningham and Fink 1996).

The vacuolar Ca^{2+}/H^{+} exchanger of *S. cerevisiae* Vcx1, also known as Hum1 for "high copy number undoes manganese", was identified for its ability to confer tolerance to high Ca^{2+} and Mn^{2+} concentrations when it is overexpressed (Pozos et al. 1996). The contribution of Vcx1 to vacuolar Ca^{2+} uptake and tolerance are markedly increased when calcineurin is inactivated, indicating that calcineurin negative controls Vcx1 activity *in vivo*, but the mechanism of this inhibition is not yet known (Cunningham and Fink, 1996; Pozos et al., 1996)

Furthermore, biochemical data using purified vacuoles and vacuole membrane vesicles have evidenced that Ca^{2+} transport activity of Pmc1 and Vcx1, more dramatically for the latter, depends on the pH gradient. Calcium uptake is promoted when the interior of these compartments is acid and lost when it is alkaline. This optimal acidification of vacuoles is maintained by the vacuolar H^{+} V-ATPase activity (Dunn et al. 1994).

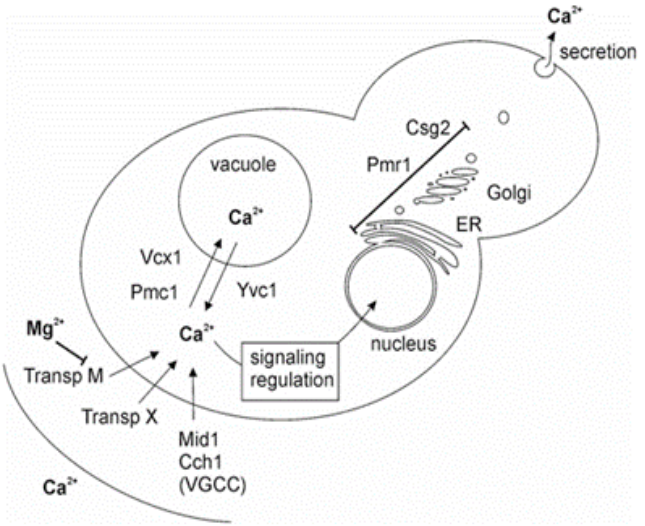

Fig. 3 Main transport elements of calcium in *S. cerevisiae* (Espeso, 2016).

The Golgi apparatus, together with vacuole, is another important calcium store in yeast cells. Calcium is concentrated in this

compartment mainly through the activity of Pmr1, a Ca^{2+}/Mn^{2+} pump homologue to the mammalian SPCA1. Pmr1 localizes primarily to the Golgi complex like its homologs in mammals (Antebi and Fink, 1992;Uccelletti et al., 1999), and therefore supplies Ca^{2+} and Mn^{2+} to the endoplasmic reticulum during its early biogenesis or through vesicle mediated trafficking in the retrograde direction from the Golgi complex.
As in high eukaryotic cells, the transport of calcium and manganese to ER is also important for the proper functioning of ER, as for the normal secretion of proteins, protein maturation and/or degradation. Thus, mutants of *S. cerevisiae* that lack the Pmr1 exhibit a range of secretion defects, as well as defects in N- and O-glycosylation (Durr et al. 1998).

1.2.3. Calcium release

About 10% of the total intracellular calcium can be released back to the cytoplasm and it is designated as the "exchangeable" pool of calcium (Cunningham and Fink 1994).
In vacuoles, calcium is present in two forms: a free and a non-usable pool. The latter is designated as "non-exchangeable" since calcium is associated to polyphosphates. The concentration of free calcium in vacuoles is about 30 μM meanwhile the total calcium was estimated at the millimolar range (2 mM) (Dunn et al. 1994). However, this reduced free-calcium pool can be returned to the cytoplasm when required.
The Ca^{2+} released from the vacuole occurs mainly via Yvc1. This channel is well conserved among fungi and it is most closely related to the TRPC-family of Ca^{2+} channels that are well characterized in animals (Palmer et al., 2001). Yvc1 is localized almost exclusively to the vacuole membrane of *S. cerevisiae* and its activation is important in response to hyperosmotic shock, reducing agents, and it has the ability to pass Na^{+} and K^{+} in vitro in addition to Ca^{2+} (Dong et al., 2010). These properties suggest that Yvc1 may be capable of rapidly releasing cationic osmolytes into the cytoplasm upon hypertonic shock to contrast the cytoplasmic dehydration and osmotic imbalance.

For the Ca^{2+} release from ER and Golgi, the current hypothesis is that calcium ions leave these compartments by exocytosis. Indeed, the genes coding for inositol tri-phosphate (IP3) receptors and ryanodine receptors, RyR, are absence in the yeast genome and calcium transporters for this activity are unknown. Moreover, it has been measured that when the calcium concentrations in Golgi and ER exceed their resting levels of 300 μM and 10 μM, respectively, the ion will be secreted along with the canonical secretory pathways (Pinton et al. 1998; Aiello et al. 2002).

1.2.4. Calcium signalling

Yeast cells are able to adapt to large and rapid modifications in environmental calcium, ranging from a low concentration of 1μM to more than 100 mM (Anraku et al. 1991) due to the activation of a signaling cascade leading to the modification of the gene expression. When calcium enters in the cytoplasm, upon elevation of extracellular levels, one of the targets is the small and essential sensor protein calmodulin, Cmd1 (Cyert, 2001). Calmodulin has many roles in the yeast cells that can be classified into calcium independent and dependent functions (Davis et al. 1986). Among the calcium-dependent roles, one it is to activate the protein phosphatase 2B calcineurin (Fig. 4). In *S. cerevisiae* calcineurin is composed of a catalytic subunit, the A subunit encoded by either *CMP2* or *CNA1* isoforms (Cyert et al. 1991), and the regulatory B subunit encoded by *CNB1* (Cyert and Thorner 1992). Activity of Cnb1, and subsequently of the calcineurin, is modulated by the α-arrestin Aly1 (O'Donnell et al. 2013) and through myristoylation (Connolly and Kingsbury 2012) in response to a reduction in calcium signal.

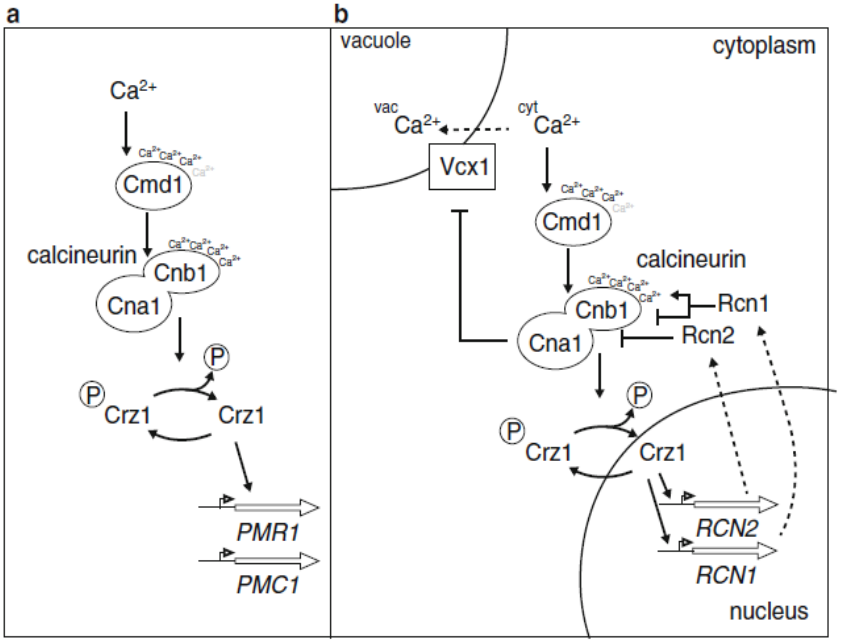

Fig. 4 Calcium signaling cascade in *S. cerevisiae*. (**a**) The calmodulin/calcineurin cascade towards activation of the transcription factor Crz1. Among Crz1 targets are the genes *PMR1* and *PMC1*, coding for the P-type ATPases, and (**b**) calcineurin regulators *RCN2* and *RCN1* (Espeso, 2016).

The calcineurin phosphatase may have numerous targets and one of these is the Crz1 transcription factor (Stathopoulos and Cyert 1997). Crz1 is a three zinc finger transcription factor that is inactivated and activated in a cyclic phosphorylation/dephosphorylation process (Dalal et al. 2014).

Crz1 mediates yeast tolerance to high concentrations of different cations, alkalinity and other types of stresses (Cyert 2003). When Crz1 is in its phosphorylated state, it is recognized by the exportin Msn5p that brings it in the cytoplasm (Boustany and Cyert 2002). After calcineurin dependent dephosphorylation in response to an elevation of the cytoplasmic calcium concentration, the nuclear import signal of Crzy is recognized by the nuclear transporter Nmd5 and the

transcription factor is translocated in nucleus (Polizotto and Cyert 2001).

In the nucleus Crz1 will bind to specific DNA sequences known as CDREs (Calcineurin-Dependent Regulatory Elements) present in the promoter of genes under its regulation (Mendizabal et al. 2001; Stathopoulos and Cyert 1997). Inducible targets of Crz1 include CRZ1 that encodes Crz1 (Matheos et al., 1997), RCN1 and RCN2 that encode positive and negative regulators of calcineurin (Mehta et al.,. 2009), *PMC1*, *PMR1*, and *ENA1* that encode several P-type cation pumps responsible for efflux of Ca^{2+}, Mn^{2+}, Na^{+}, and Li^{+}, cell wall biosynthetic enzymes (*FKS2*) and many other genes that control other processes (Yoshimoto et al., 2002).

2. Yeast as a model organism

Yeasts are eukaryotic unicellular organisms belonging to the kingdom Fungi. With an ovoid or spherical shape, the size of yeast cells typically measure 3–4 µm in diameter, although some yeasts can grow to 40 µm in size (Walker et al., 2002).
Yeasts do not form a single taxonomic or phylogenetic group, indeed they are placed into two separate phyla: the Ascomycota (like *Saccharomyces cerevisiae* and *Kluyveromyces lactis*) and the Basidiomycota (Kurtzman, 1994).
Some of the properties that make yeast suitable for biological studies include rapid growth, dispersed cells, the ease of mutant isolation, a well-defined genetic system, and most important, a highly versatile DNA transformation system. The useful physiological properties of yeasts have led to their use in the field of biotechnology; fermentation of sugars; moreover, yeast represents one of the most widely used model organisms for genetics and cell biology (Fig. 5).

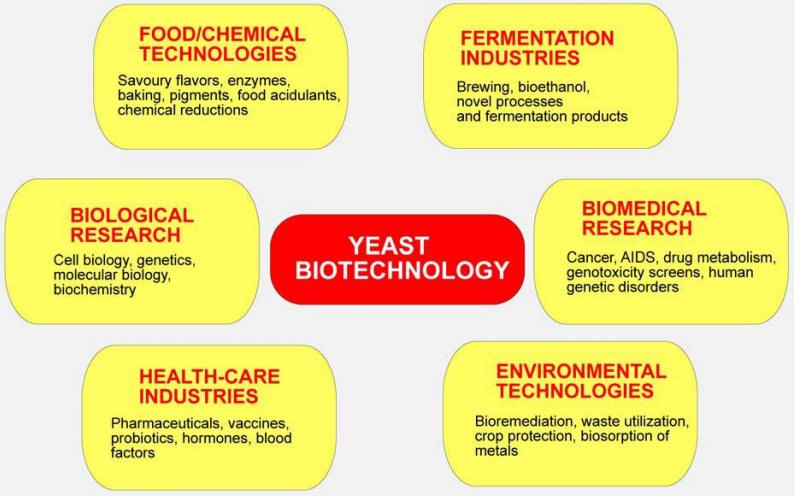

Fig. 5 Biotechnology fields involving yeast. (Tenreiro & Outeiro, 2010).

An important theme of yeast biology is the use and study of homologous recombination, a process by which a broken piece of DNA

uses a homologous DNA template as a substrate for repair. Yeast use this process to fix DNA damage, to switch mating types, and to segregate homologous chromosomes during meiosis. Researchers also use this pathway for genetic mapping and integrative transformation of DNA into specific locations of the genome. Much of what we have learned about cell and molecular biology has come from research on yeast. The genes and mechanisms involved in many cellular processes are highly conserved across eukaryotic taxa, so by studying the yeast we learn about the fundamental biology of all eukaryotes (Mell and Burgess, 2002).

The yeast *Saccharomyces cerevisiae* is commonly known as baker's yeast or brewer's yeast and it is the most extensively characterized eukaryotic organism. Yeast cells divide about every 90 minutes under optimal laboratory conditions, through a process of budding in which smaller daughter cells pinch, or bud, off the mother cell. The common name "budding yeast" derives from this notable feature. In the haploid cell, *S. cerevisieae* genomic DNA is subdivided into 16 chromosomes and according to the SGD report of February 2014 (http://www.yeastgenome.org/), the "verified open reading frame (ORFs)" in the reference strain S288C stood at 5076 (Duina. et al., 2014).

Several features of yeast have been instrumental for our current understanding of conserved cellular mechanisms such as cell division, DNA replication, metabolism, protein folding and intracellular transport (Fields and Johnston, 2005). With a cell cycle that involves haploid and diploid forms, the study of lethal mutations in heterozygous diploids and recessive mutations in haploids is extremely simplified in yeast. Furthermore, classical genetic manipulations are facilitated by mating the haploid strains and sporulating diploid strains. Moreover, the high transformation efficiency of these cells and the presence of a very efficient homologous recombination render relatively easy to insert, delete or mutate any genomic sequence up to the chromosome level (Sugiyama et al., 2009). Because of the advantages described above, *S. cerevisiae* was the first eukaryotic organism to be fully sequenced in 1996 (Goffeau et al., 1996).

At the moment, several biological resources exist for the yeast researchers. They include the yeast gene deletion strains (YGDS), where every protein-coding gene in the genome was deleted (Winzeler

et al., 1999); the green fluorescent protein (GFP)-tagged collection of strains, where each gene was tagged with GFP (Huh et al., 2003); as well as a collection of plasmids for protein overexpression (Jones et al., 2008). Additionally, high-throughput data obtained from functional genomics approaches, such as transcriptomics, proteomics and metabolomics are available.
Notably, information about predicted orthologs in humans is also organized and available for each yeast gene (Tenreiro e Outeiro, 2010)

2.1. Yeast as model of human disease

Most of the basic cellular functions are conserved from yeast to humans as well as the diseases's key players are often evolutionary conserved. Indeed, 31% of the yeast genes have a mammalian homologue and an additional 30% of yeast genes show domain similarity (Botstein et al., 1997). Consistently, about 30% of the genes known to be involved in human diseases may have a yeast orthologue (Botstein et al., 1997; Foury, 1997). For these reasons, such as for its versatile genetic flexibility, yeast has been increasingly used as a model and tool for biomedical research over the past one or two decades (Mager and Winderickx, 2005).
Yeast models of human diseases enable identification and characterization of key actors and possible therapeutic targets associated to considered disorders. Moreover, this simple organism is largely used to high-throughput genetic and small-molecule screens to find possible pharmacological drugs.

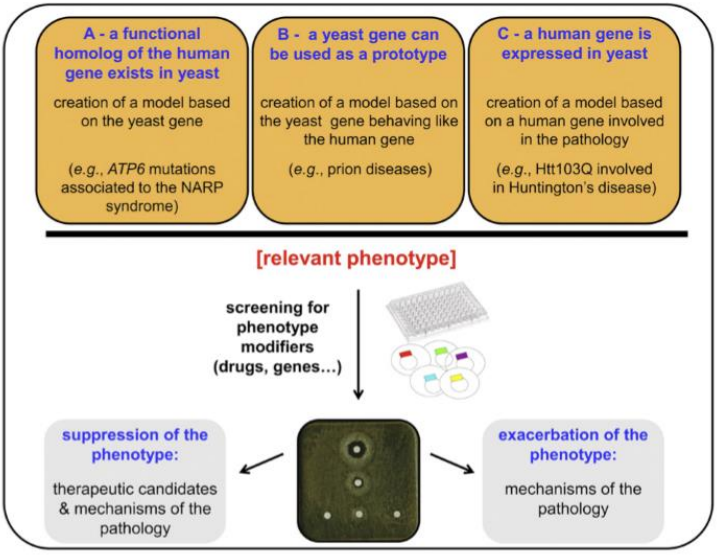

Fig. 6 The uses of yeast as model for human diseases (Voisset et al., 2014).

In order to create a proper yeast model for a given human pathology, it is first necessary to get a phenotype, which is relevant close to the known pathological mechanism and easy to follow using classical yeast tools.

2.1.1. Yeast modelling for mitochondrial disorders

In the simplest cases, where a functional homolog of the affected protein in human exists in yeast, the mutated forms of the human mutant proteins can be expressed directly in yeast strains in which the corresponding yeast gene has been deleted. Hence, the use of yeast to determine the functionality of mutated alleles of genes involved in genetic diseases has been largely exploited in the field of mitochondrial diseases. Indeed, much of our current understanding of mitochondrial function and dysfunction comes from studies in the baker's yeast

Saccharomyces cerevisiae. Because of its good fermenting capacity, *S. cerevisiae* can survive mutations that inactivate oxidative phosphorylation, it has the ability to tolerate the complete loss of mitochondrial DNA (a property referred to as 'petite-positivity'), and is amenable to mitochondrial and nuclear genome manipulation (Lasserre et al., 2015).
For example, yeast model was used to investigate the effects of mutations in the mitochondrally- encoded gene *ATP6*. Mutations in this gene causes a devastating mitochondrial disease, the NARP (Neuropathy, Ataxia, Retinitis Pigmentosa) syndrome (Couplan et al., 2011). The expression of a mutated human form of *ATP6* (T9176G) in yeast severely was found to impede the incorporation of encoding subunit 6 in the yeast ATP synthase (Kucharczyk et al., 2010), and evidences for similar defects have been reported in skin fibroblasts from patients carrying this mutation (Carrozzo et al., 2001). Another approach introduced the identified mutations of human *ATP6* gene in the yeast homologue to identify genes and drugs that suppress their respiratory growth phenotype (Couplan et al., 2011). These yeast-NARP models were further validated when these drugs were shown to also suppress the respiratory growth of cells derived from NARP patients.

2.1.2. Yeast gene as prototype

In the absence of a functional ortholog, yeast models can still be created by using yeast genes as "prototypes". This approach has been performed for prion-related diseases. Indeed, although they present no homology with the mammalian prion PrP, yeast prions themselves were used as models for PrP, based on the initial assumption that prion-controlling mechanisms may be conserved throughout evolution. Moreover, like their mammalian counterpart, yeast prions form autocatalytic amyloid fibers, which are partially resistant to proteinase K. A yeast assay was therefore developed to isolate antiprion compounds based on their activity against two unrelated yeast prions (Tribouillard et al., 2006). The isolated active molecules then turned

out to be active against mammalian prion both in cell-based assays and *in vivo* in a mouse model for prion diseases (Oumata et al., 2013). Importantly, this study constitutes the first functional evidence that at least some cellular mechanisms involved in prion propagation are conserved from yeast to human.

2.1.3. Yeast model for nuerodegenerative disorders

When neither functional orthologue nor prototype gene exists in yeast, human disease models can be created by heterologous expression of the human mutated protein in yeast. This type of approach has been largely used in the field of neurodegenerative diseases like Huntington's (Giorgini et al., 2005; Zhang et al., 2005), Parkinson's (Outeiro and Lindquist, 2003) and Alzheimer's disease (Fig. 7).

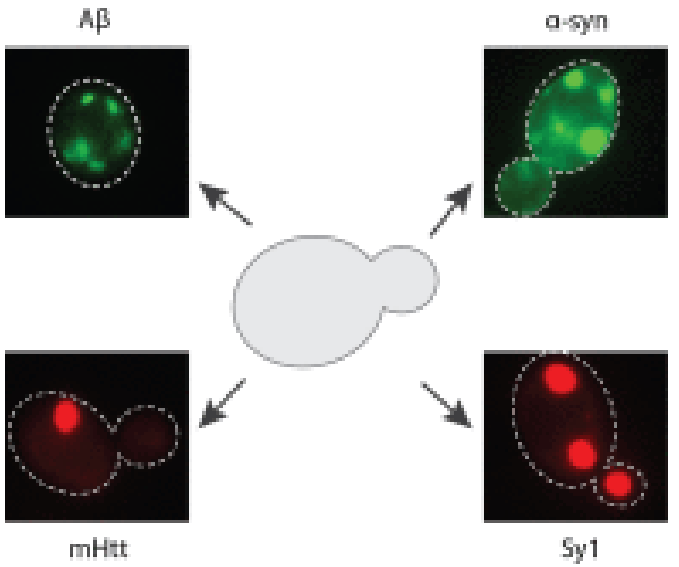

Fig. 7 Yeast as model of neurodegenerative disease (Fruhmann et al., 2016).

Although the human proteins involved in these diseases have no functional homolog in yeast, upon expression in this organism they form protein aggregative fibers, the hallmark of these disorders, that can even, at least in some cases and/or situations, be toxic for yeast cells, thereby mimicking the situation observed in human neuronal cells (Outeiro and Lindquist , 2003).

For the Huntington's disease, a mutant form of the first exon of Huntingtin containing the expanded polyglutamine domain (103Q instead of 25Q in the wild type allele) was expressed in budding yeast, where the encoding protein aggregated and was toxic as in patients' neuronal cells (Giorgini et al., 2005). Moreover, using a collection of haploid strains deleted for every single non-essential yeast genes was found that aggregates of Htt103Q were not toxic in some yeast mutants, like in the *bna4* mutant. This discovery suggested that the Bna4 gene, which encodes kynurenine 3-monooxygenase (KMO), an enzyme of the kynurenine pathway of tryptophan degradation highly conserved from yeast to humans, is a putative therapeutic target for the treatment of Huntington's disease. This hypothesis was later on pharmacologically validated using chemical inhibitors of KMO in animal models for both Huntington's and Alzheimer's diseases (Zwilling et al., 2011).

Mutations in α-synuclein, causing Parkinson's disease (PD), were also expressed successfully in yeast, where mutated protein accumulated into cytoplasmic inclusions like in human neurons (Outeiro and Lindquist, 2003).

Furthermore, Cooper and co-workers demonstrated that in yeast α-synuclein aggregates impair vesicle transport from the ER to the Golgi. In the same study, a genome-wide overexpression screen identified the small GTPase Ypt1 as a modifier of α-synuclein toxicity. Overexpression of Ypt1p was sufficient to prevent α-synuclein toxicity, by enabling forward trafficking from the ER to the Golgi. This observation was further extended to *Drosophila* and *C. elegans* models of PD, as well as in rat midbrain primary neurons, where Rab1—the functionally ortholog of Ypt1p— suppressed dopaminergic neuron loss that characterized PD patient (Cooper et al., 2006).

Finally, yeast is still a powerful model to study Alzheimer's disease (AD), characterized by the presence in neurons of intracellular neurofibrillary tangles (NFT) of Tau protein and extracellular plaques

of β-amyloid (Aβ) (Guo and Lee, 2014;Lee et al., 2001). Among the yeast studies of Alzheimer, one in 2011 identified different modifiers of β-amyloid toxicity that are conserved from yeast to humans (Treusch et al., 2011). One of these modifiers was YAP1802, a suppressor of β-amyloid proteotoxicity that is involved in clathrin-mediated endocytosis. Its human homolog PICALM is also involved in endocytosis and has been validated as a high-risk factor for AD. YAP1802 prevents β-amyloid toxicity in yeast and PICALM prevents β-amyloid toxicity in rat cortical neurons. Notably, this study identifies a causal gene for susceptibility to AD and proposes defective endocytosis as a contributing factor in AD pathology, with a possible role for PICALM.

Therefore, in all of these models, the basic idea is always obtaining a yeast phenotype that is relevant for the considered disorders which then offers the possibility to search for modifiers that either suppress or exacerbate a given phenotype. Modifiers can include drugs, genes or any other biological or chemical moiety.

3. The yeast model of Hailey Hailey disease

3.1. Hailey Hailey disease

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 and with an incidence of 1:50000 (Vasudevan et al., 2015). The name comes from the first described case by two brothers William Howard Hailey and Hugh Edward Hailey, in 1939. The two siblings had recurrent bullae and erosion on their neck regions, and they referred to the pathology as chronic erosive bullous dermatosis of flexures (Engin et al., 2015).

The genetics and pathophysiology of HHD have been linked to mutations in the ATP2C1 gene (Sudbrak et al., 2000; Hu et al., 2000).

The gene, located on the long arm of chromosome 3; 3q21-q24 region, encodes the human secretory pathway Ca^{2+}/Mn^{2+} ATPase, hSPCA1 (Wuytack et al., 2002).

This pump is ubiquitously expressed in human tissues but with the greatest abundance in keratinocytes (Hu et al., 2000). Although, hSPCA1 is mostly localized to the Golgi apparatus, it detectably contributes to filling up ER Ca^{2+} stores in certain cell types and has been shown to exert effects on both Golgi and ER functions.

The contribution of hSPCA1 to the total Ca^{2+} uptake of the Golgi varies among different cell types from 15% to 50%; however, in keratinocytes, the role of SPCA1 is even more significant (Missiaen et al., 2004). This in part may explain why only the skin is affected in HHD, although the exact reason for this phenomenon is currently not well understood. One hypothesis is maybe because calcium has important regulator function in keratinocyte proliferation and differentiation (Yuspa et al., 1988).

More than 100 different mutations in ATP2C1 have been reported in HHD. Among these 20% are nonsense mutations, 19% are splice site mutations, 30% are frameshift mutations leading to premature termination codons (PTC), 28% are missense mutations, and ∼3% are in-frame deletions or insertions (Kellermayer, 2005).

The high number of PTC mutations suggests the haploinsufficiency as the prevalent mechanism for the dominant inheritance of HHD. Studies

on missense mutations shown that while mutant ATP2C1 mRNA levels are normal, the level of the defective protein is lower than that of the wild type despite correct targeting to the Golgi, supporting the thesis of haploinsufficiency.
Interestingly, the expression of ATP2C1 was downregulated in cells derived from lesional skin areas of HHD patients but not in keratinocytes originating from unaffected skin areas of the same patient (Cialfi et al., 2010).
The lack of ATP2C1 impairs keratinocytes ability to store Ca^{2+} normally and this leads to the loss of cell-to-cell adhesion (called acantholysis) among the cells of the suprabasal layer of epidermis probably due to a retraction of keratin intermediate filaments from the desmosomal plaques (Dobson-Stone et al., 2002).
In the healthy epidermis, a Ca^{2+} gradient is present with lowest calcium levels in the basal layer and highest in the granular layer of the skin both intra and extracellularly. This gradient appears to be a key element for the keratinocyte differentiation, in which the calcium sensing receptor (CaR) is an important player (Elias et al., 2002; Tu et al., 2001).
In HHD epidermis, both in the lesional and intact skin, this calcium gradient is absent, likely due to the inability of HHD keratinocytes to accumulate calcium properly upon increasing extracellular levels of Ca^{2+}, that occurs for example after noxious stimuli. While total calcium levels are significantly decreased in the granular layer of the HHD, the basal and suprabasal layers contain similar amounts of calcium as the healthy skin (Hu et al., 2000). These observations support the idea that the primary defect in HHD skin is the decreased amount of total calcium in the granular layer, although the suprabasal keratinocytes suffer from desmosomal disruption (Fig. 8). HHD keratinocytes have also normal amounts of desmosomal proteins that can bind other proteins with the same efficiency as seen in normal epidermal cells; moreover, desmosome formation and response to UVB irradiation are similar between HHD and normal keratinocyte cultures. These observations indicate that the whole changed environment of the epidermis is required for the alteration in the HHD keratinocyte desmosome assembly.
In addition, altered Ca^{2+} homeostasis in HHD keratinocytes seems to induce an early defect in differentiation process due to a reduced

production of involucrin, a protein that makes up the cornified envelope of keratinocytes and that is expressed in response to increased intracellular Ca^{2+} concentrations (Aberg et al., 2007).
Some specific mRNAs and oxidative stress may have roles in the pathogenesis of HHD. Indeed oxidative stress that characterized HHD keratinocytes leads an increased expression of the miR-125b. In lesional-derived keratinocytes of HHD patients, this mRNA seems to be involved in the down-modulation of notch1 and p63, two key factors in the keratinocyte proliferation and differentiation (Cialfi et al., 2010; Manca et al., 2011).

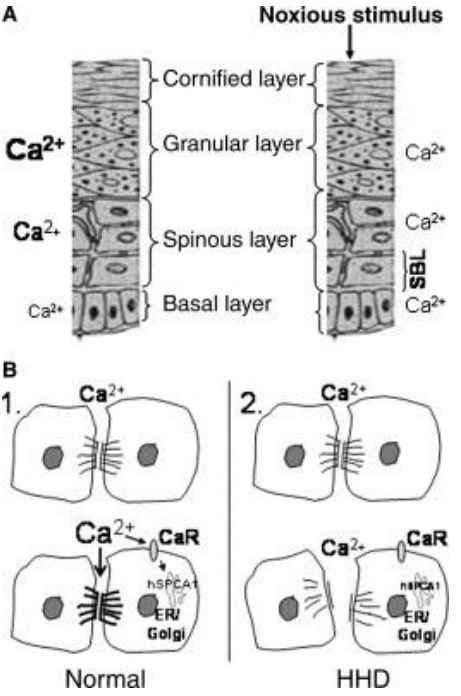

Fig. 8 Suprabasal acantholysis in HHD keratinocytes due to the altered calcium homeostasis (Elias et al., 2002)

As a result, keratinocytes do not stick tightly to one another, so the epidermis becomes fragile and less resistant to minor trauma. Because the skin is easily damaged, it develops raw, blistered areas, particularly in skin folds where there is wetness and friction (Fig. 9). Indeed the areas mostly affected are neck, axillae and groins.

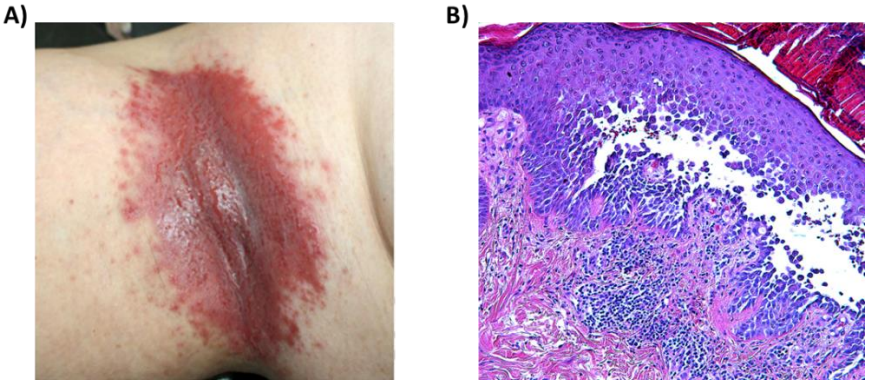

Fig. 9 (A) Erythematous plaque with multiple fissures on axillary region (Engin et al., 2015). **(B)** Suprabasalar and intraepidermal keratinocyte acantholysis (Graham et al., 2016).

The disease has a fluctuating course with remissions and exacerbations and in rare instances, the rash may become generalized (Chave and Milligan, 2002); rarely, skin lesions may develop into squamous cell carcinomas (Chun et al., 1988; Cockayne et al., 2000) or melanomas (Mohr et al., 2011).
Important factors that can trigger the manifestation of the pathology are: menstruation, pregnancy, skin infections, physical trauma, excessive sweating and exposure to ultraviolet radiation.
At present, there is no reported cure for HHD and current treatments are not particularly effective in all patients. The treatment is primarily aimed at symptomatic relief. Since superinfection with bacteria, fungi, and viruses plays an important role in exacerbations and persistence of lesions, topical antibiotics, antifungal and antiviral agents are

commonly used in the HHD lesion treatments. Moreover systemic, topical, and intralesional corticosteroids have been found to be useful in the management of HHD in many cases (James et al., 2006; D'Errico et al., 2012)
Other drugs that have also proved to be effective in some cases are cyclosporine, retinoids, botulinum toxin A, and dapsone **(**Bagherani and Smoller, 2016; Engin et al., 2015). Particularly, the botulinum toxin A seems to be effective by decreasing sweat and subsequent microorganism colonization of lesion (Bessa et al., 2010). Meanwhile, ablative lasers give better results with the recalcitrant plaques of HHD patients (Ortiz and Zachary, 2011).

3.2. Yeast as model of HHD

As described before, few is known about the molecular factors and pathways involved in HHD pathogenesis; moreover, therapies are not available. Moreover, a mouse model does not exist. In fact, meanwhile null mutant Spca1−/− embryos did not survive to the gestation, heterozygous mice for ATP2C1 do not develop the typical vesicular lesions of human patients. Indeed, they have only an increased incidence of squamous cell tumors (Okunade et al., 2007).
As mentioned above, in the last 10-20 years the yeast, in particular *S. cerevisiae*, has become an effective and simple model system to understand the molecular players associated to a given pathology.
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 et al., 1999; Rudolph et al., 1989). Expression of the human ATP2C1 in yeast fully rescues the yeast *pmr1* hypersensitivity to Ca^{2+} chelators and Mn^{2+} toxicity (Ton et al, 2002). Although ATP2C1 shares only 49% amino acid sequence identity with Pmr1, the transmembrane domains known to be important for the ion transport are almost completely conserved. This observations validated the use of yeast *PMR1* gene to study some aspects of ATP2C1 function, to determinate the functionality of mutated alleles of ATP2C1 found in

HHD patients and then to explore the pathophysiology and potential therapy of this genodermatosis (Mauro, 2004; Ton and Rao, 2004). Pmr1p is responsible for maintaining Ca^{2+}/Mn^{2+} homeostasis in both Golgi apparatus and ER. Although it is not essential, cells deprived of Pmr1p display pleiotropic phenotypes, some of them resembling those reported in HHD keratinocytes, including alterations in Ca^{2+} homeostasis, mitochondrial alterations and ROS accumulation (Hu et al. 2000; Uccelletti et al., 2005).

Moreover, as a result of Ca^{2+} and Mn^{2+} deficiency within the ER and Golgi, the *pmr1Δ* mutant is defective in protein sorting and processing through the secretory pathway (Durr et al., 1998; Ramos-Castañeda et al., 2005). The alterations in protein processing cause an accumulation of chitin in the *pmr1*Δ mutant. As a result the mutant cells become hypersensitive to the chitin binding stilbene fluorescent brightener calcofluor white (CFW). This phenotype of *pmr1Δ* can be used to screen drugs that could have therapeutic effects on HHD. For example Voisset et al., used this CFW-phenotype of the *pmr1*Δ mutant to screen two different chemical libraries, looking for suppressors of the CFW-sensitivity (Voisset et al., 2014). However, the molecular mechanisms of these compounds are unknown and they need to be validated on human cell-based models of Hailey–Hailey disease.

Furthermore, studies in *S. cerevisiae* elucidate the action mechanism of tacrolimus (FK506) and cyclosporine, two immunosuppressors used in some case of recalcitrant Hailey–Hailey (Rabeni and Cunningham, 2002; Varada et al., 2015). These two compounds are calcineurin inhibitors and although their major mechanism of action is presumably the suppression of lymphocyte activation, a non immunomodulatory mechanism has also been proposed. Studies on the effects of calcineurin inhibition in *S. cerevisiae pmr1Δ* mutants showed that cyclosporine induced tolerance to high extracellular Ca^{2+} environment through vacuolar Ca^{2+}/H^{+} exchanger activity of Vcx1 (Szigeti and Kellermayer, 2004). Moreover, FK506 (tacrolimus) has been found to augment Vcx1 function in *pmc1Δ* yeast under Ca^{2+} stress (Cunningham and Fink, 1996). Although calcineurin has been shown to be functionally active in human HHD keratinocytes (Al Daraji et al, 2002), the observations in yeast suggest that calcineurin inhibitors such as tacrolimus and cyclosporine may be beneficial in the treatment of Hailey–Hailey disease by decreasing Ca^{2+} stress in epidermal cells.

Other studies showed that yeast is a powerful model organism for Hailey Hailey disease. Indeed, in the yeast *Kluyveromyces lactis* the loss of *PMR1* provokes an increased ROS production compared to wild type cells (Uccelletti et al., 2005),as well as keratinocytes derived from lesional skin show an increased ROS levels compared to non lesional-derived ones of the same HHD patients (Cialfi et al., 2010)

4. The yeast *Kluyveromyces lactis*

Kluyveromyces lactis is both scientifically and biotechnologically one of the most relevant non-*Saccharomyces* yeasts. Its biotechnological significance builds on its history of safe use in the food industry and its well-known ability to produce enzymes like lactase and bovine chymosin on industrial scale (Faraco et al., 2008; van Ooyen et al., 2006).
This microorganism grows in optimal temperature at 25°C to 40°C and the cells have oval form and typically measure 3–4 µm in diameter (Fig. 10).

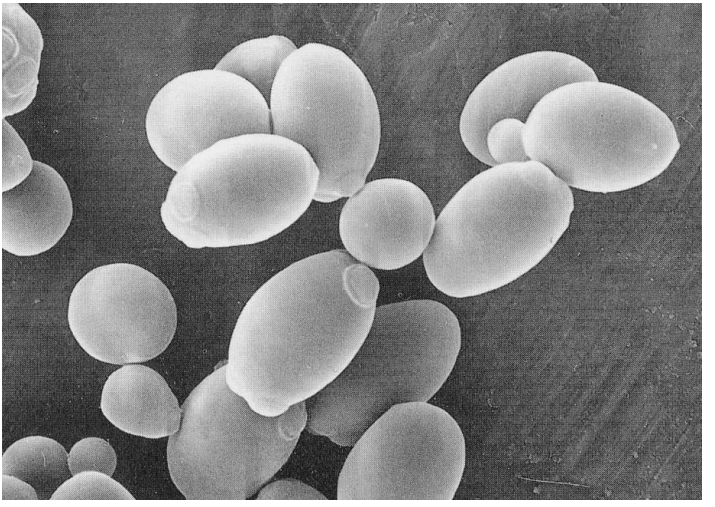

Fig. 10 *Kluyveromyces lactis* cells.

Its genome is divided up into 6 chromosomes ranging in size between 1 Mbp and >3 Mbp.
Because *K. lactis* can ferment lactose it is mainly used in the dairy industry. Some of the most important applications include the industrial production of native β-galactosidase and recombinant milk clotting enzyme bovine, chymosin. This protein was the first heterologous

enzyme originating from a higher eukaryote that was produced at low cost in a microorganism, and the process developed for its industrial-scale production was widely recognized as a major biotechnological achievement (van den Berg et al., 1990).
Moreover, the enzyme β-galactosidase is used to produce lactose-free dairy products and prebiotic galacto-oligosaccharides (GOS) (Alves Macedo et al., 2003; Guerrero et al., 2015).
Given its successful applications in the dairy industry, *K. lactis* was later introduced as a host for the large-scale production of heterologous proteins in the pharmaceutical industry. One example is the production of human interleukin-1β, which is used to treat autoimmune disorders (Fleer et al., 1991; Maedler et al., 2009).
K. lactis has a number of advantages over other yeast expression systems, including easy genetic manipulation, the ability to use both integrative and episomal expression vectors, and the availablility of a fully sequenced genome (Dujon, 2004). Growth of *K. lactis* cells can be performed in standard yeast medium and does not require the explosion-proof fermentation equipment necessary for large-scale growth of methylotrophic yeasts such as *Pichia pastoris*. In addition, enzymes from *K. lactis* have GRAS (Generally Regarded As Safe) FDA status, permitting their use in various food and feed applications (van Ooyen et al., 2006).
K. lactis, like *S. cerevisiae*, also facilitates the investigation of basic cellular processes. The existence of different protein isoforms makes the analysis of *in vivo* protein functions or complementation assays with human proteins challenging (Spohner et al., 2016). Unlike *S. cerevisiae*, *K. lactis* has not undergone whole genome duplication and most enzymes and other proteins occur as single isoform. *K. lactis* is therefore a more suitable model than other yeasts for the investigation of human diseases and cellular defence mechanisms (Bharadwaj et al., 2010; González Siso and Cerdán, 2012).
Finally, the regulatory circuits of *K. lactis* responses to oxygen limitations have stronger resemblance in humans, compared to *S. cerevisiae* redox metabolism. Indeed, *K. lactis* is characterized, opposite to *S. cerevisiae*, by a higher glucose flow through the pentose phosphate pathway (PPP) than through glycolysis. Thus, *K. lactis* is proposed as a respiratory eukaryote model, complementary to the fermentative *S. cerevisiae*, for the study of the pathways of hypoxia-

induced oxidative stress, which are related to human diseases such as Alzheimer's and Parkinson's (Gonzalez Siso and Cerdan, 2012).

AIM OF THE PROJECT

Over the past one or two decades, yeast has been increasingly used as a model and tool for biomedical research (Mager and Winderickx, 2005; Perocchi et al., 2008). The reasons lie in the unique characteristics of this eukaryotic unicellular organism. Indeed, it is experimentally tractable with a rapid doubling time and it grows in very simple culture conditions; yeast is thus a eukaryote easy to handle as a prokaryote.
In addition, numerous powerful tools are available when working with this simple model, like classical genetics, cell biology, biochemistry and molecular genetics. Indeed the genome of budding yeast *S. cerevisiae* was the first eukaryotic genome to be sequenced (Goffeau et al., 1996) and it can be modified at will at the nucleotide level. Therefore, all its essential genes have been identified.
Furthermore, a huge number of systematic approaches (transcriptomic, proteomic, locabolomic, interactomic, metabolomic, etc.) have been carried out in yeast and their results are freely available. Thanks to the richness of this molecular toolbox, yeast has long been used as a powerful model system for basic research on the most fundamental cellular mechanisms, like mitochondrial functions, transcription, translation, DNA replication or secretion.
Most of the basic cellular functions are conserved from yeast to humans as well as the diseases's key players are often evolutionary conserved. Indeed, about 30% of the genes known to be involved in human diseases may have a yeast orthologue (Botstein et al., 1997; Foury, 1997). For these reasons, such as for its versatile genetic flexibility, yeast is used as a model and tool to investigate the molecular mechanism and players involved in human disease (Mager and Winderickx, 2005).
Besides, this simple organism is largely used to high-throughput genetic and small-molecule screens to find possible pharmacological drugs.

Hailey–Hailey disease (HHD), also known as familial benign chronic pemphigus, is a rare, chronic and recurrent blistering disorder. It is histologically characterized by the lost of contact among the keratinocytes of suprabasal layer of skin (acantholysis). The genetics and pathophysiology of HHD have been linked to mutations in the

ATP2C1 gene encoding for hSPCA1, a Ca^{2+}/Mn^{2+}-ATPase pump (Hu et al, 2000).
At the moment, few is known about the molecular players involved in HHD pathogenesis.
Moreover, mouse does not represent a good model for this disorder. In fact, whereas the Spca1−/− embryos do not survive to the gestation, in the heterozygous mice for ATP2C1 the typical vesicular lesions are absent. Instead, they have an increased incidence of squamous cell tumors (Okunade et al., 2007) that are very rare in the HHD patients.
In yeast, *PMR1* is the ortologue gene of ATP2C1. Expressing the human ATP2C1 in yeast *S. cerevisiae* fully rescues the yeast *pmr1* hypersensitivity to Ca^{2+} chelators and Mn^{2+} toxicity (Ton et al, 2002) and although hSPCA1 shares only 49% amino acid sequence identity with Pmr1, the transmembrane domains known to be important for transport are almost completely conserved.
Furthermore, in the budding yeast *Kluyveromyces lactis* the loss of *PMR1* provokes an increased ROS production compared to wild type cells (Uccelletti et al., 2005), similar to the HHD keratinocytes derived from lesional skin that showed a higher levels of ROS with respect to the no lesional-derived ones of the same HHD patients (Cialfi et al. 2010).
This and other observations indicate that yeast can be a useful simple model to study the molecular mechanism involved in the HHD pathogenesis.
For this reason, we use the genetic tractable yeast *K. lactis* to analyze the relationship between calcium and ROS production in ATP2C1/*PMR1*- deleted cells. To this aim, we will perform a genetic screening to identify molecular player able to alleviate the oxidative stress and/or the calcium alterations that characterize *pmr1Δ* cells.

Currently a specific cure for HHD patients is absent; indeed, primary treatments consist of topical antibiotics and antifungal to fight the infections that plays an important role in exacerbations and persistence of lesions. Also topical and intralesional corticosteroids have been used in the management of HHD in many cases to reduce the inflammation of the skin lesions (Voisset et al., 2014).
Thus, to extend our knowledge on compounds that improve the condition of HHD patients, in parallel with the genetic screening we

will perform a pharmacological screening. We will take advantage of the 131 FDA-approved natural compounds to analyze their ability to suppress the *pmr1Δ* phenotypes. In the initial screening system we will evaluate if the drugs are able to recover the cell wall defects of our mutant.

RESULTS

1. Glutathione S-transferase Theta-subunit and Hailey-Hailey Disease: from yeast to human keratinocytes

1.1. Glutathione S-transferase rescues the oxidative stress of *Klpmr1*-deleted cells

In a previous work, we demonstrated that the *pmr1Δ* mutant is characterized by altered mitochondrial morphology associated with increased accumulation of reactive oxygen species (ROS) (Uccelletti et al., 2005). In order to investigate the possible molecular interactions of Pmr1, we performed a genetic screening to identify multicopy suppressors able to relieve the growth sensitivity to hydrogen peroxide (H_2O_2) of cells lacking the Ca^{2+}-ATPase activity. To this aim, we transformed *Klpmr1Δ* cells with a cDNA library derived from MDCK cells (Guillen et al., 1998) and we selected the positive clones that were able to growth in the presence of H_2O_2. The screening allowed the identification of the Glutathione S-Transferase Θ-subunit (GST), an important detoxifying enzyme, as the suppressor able to completely rescue the growth defects of *pmr1Δ* cells in the presence of the stressor molecule (Fig1A). In figure 1B, it is shown the alignment of the amino acid sequence of *K. lactis* with related GSTs from *S. cerevisiae* and higher eukaryotes, e.g. human and *Canis lupus familiaris*, highlighting several regions with high degree of similarity. Based on this, a transcription analysis of the yeast gene of Glutathione S-transferase (*GTT1*) was thus performed. As shown in fig. 1C, a reduction in *GTT1* transcript was observed in the *Klpmr1Δ* mutant strain in comparison with its wild type counterpart

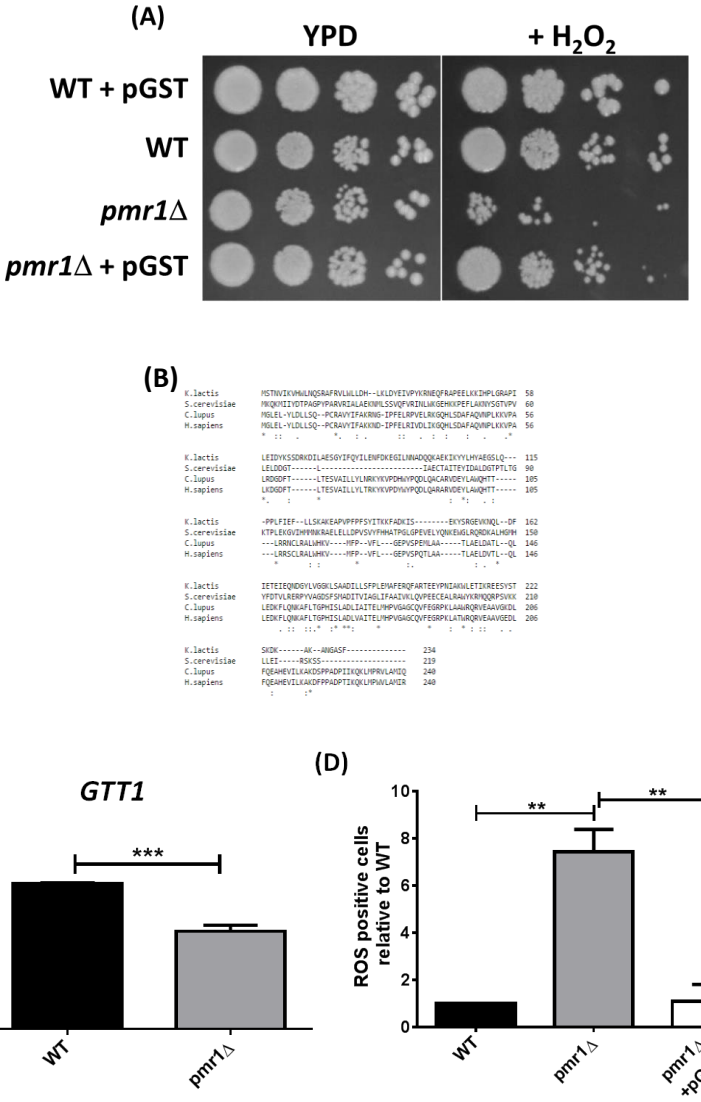

Fig. 1 (A) The genetic screening with the MDCK cDNA library allowed the identification of GST as a suppressor of the *pmr1Δ* sensitivity to H_2O_2. (**B**) Amino acid alignment of the *K. lactis*, *S. cerevisiae*, *Canis lupus familiaris* and *Homo sapiens* Glutathione S-transferase protein. Identical amino acids are indicated with a star (*****), meanwhile conservative or semi-conservative substitutions are showed with a colon (**:**) or period (**.**), respectively. **(C)** Transcript levels of *K. lactis* Glutathione S-transferase GTT1. **(D)** ROS levels in wild type (black bar), *pmr1*Δ (grey bar) and in transformed (white bar) strains.

To confirm the relief of oxidative stress by GST overexpression, we measured the ROS production of our strains using the cytofluorimetric analysis. As shown in fig. 1D, effectively, ROS accumulation was completely suppressed by enhancing Glutathione S-Transferase transcription in the mutant cells.

Next we also evaluated the transcriptional profile of different key enzymes involved in oxidative stress detoxification (Fig. 2). While no changes were observed for the cytosolic (*SOD1*) and mitochondrial (*SOD2*) superoxide dismutase genes, mRNA levels of both cytosolic (*CTT1*) and peroxisomal (*CTA1*) catalases were high in the *Klpmr1Δ* genetic background (Fig. 2). However, the increase in mRNA levels was completely abolished by GST over-expression in the case of *CTT1* gene (Fig. 2).

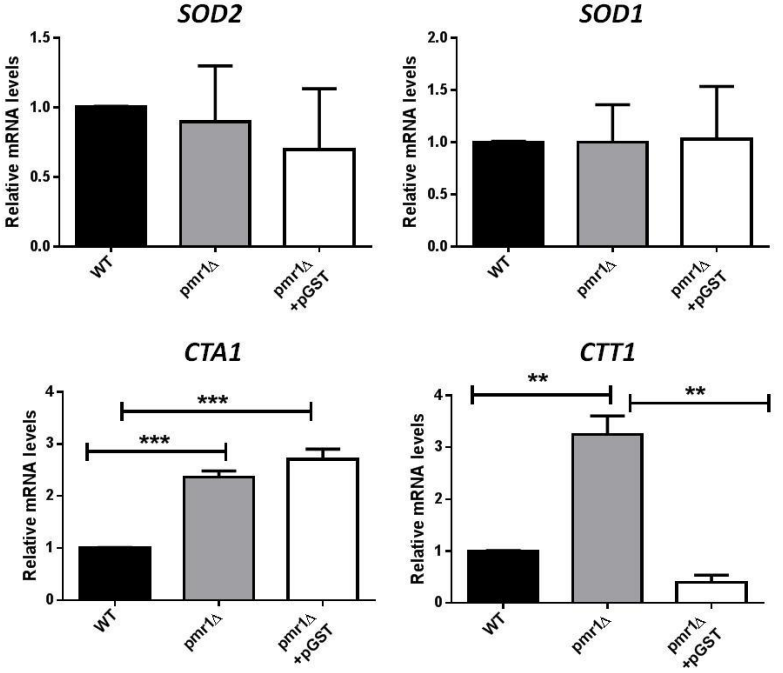

Fig. 2 Transcriptional level of superoxide desmutases and catalases in WT, *PMR1*-deleted cells and *Klpmr1Δ* + pGST

1.2. The mitochondrial dysfunction of mutant cells is recovered by GST overexpression

Since mitochondrial alterations are often associated with ROS accumulation, we wondered if GST may relieve the mitochondrial dysfunction that characterized the *KlPMR1*-deleted cells (Uccelletti et al., 2005). To this aim, *K. lactis* cells were stained with DASPMI, a fluorescent probe that is taken up by mitochondria as a function of membrane electrochemical potential. The overexpression of the mammalian GST in the mutant cells rescued the mitochondrial functionality with a full recovery of the wild type-like tubular mitochondrial network; meanwhile in the cells lacking of *PMR1* we observed the presence of dots, indicating an alteration in the mitochondrial activity (Fig. 3).

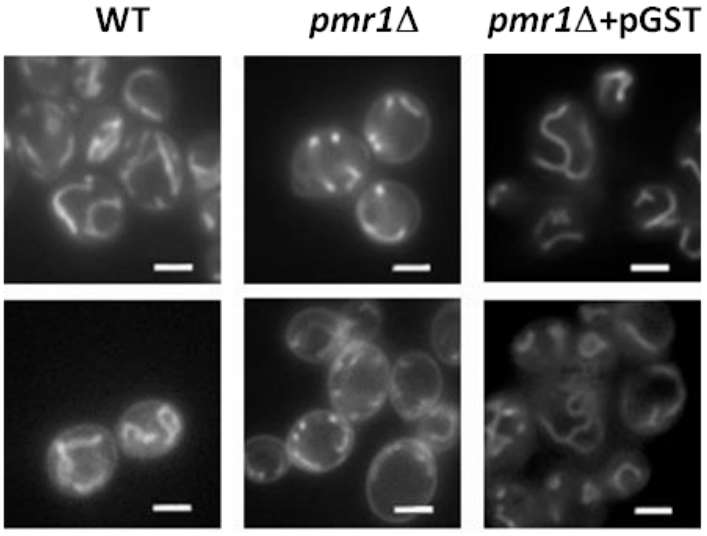

Fig. 3 Fluorescence micrographs of DASPMI-stained cells. All pictures were taken at the X100 magnification; scale bar 2 μm.

1.3. GST is able to alleviate the calcium alteration of *KlPMR1* deleted cells

Previously in our laboratory it was found that the loss of the Golgi Ca^{2+}-ATPase in *K. lactis* cells determined calcium-related phenotypes, including high levels of cytosolic calcium ($[Ca^{2+}]$cyt) (Uccelletti et al., 2005). Thus we investigated if the overexpression of mammalian GST was able to affect the calcium alterations of *pmr1Δ* cells. By cytofluorimetric analysis, we observed that Glutathione S-Transferase only partially suppressed the increased cytosolic calcium levels of the mutant strain (Fig. 4 A). Furthermore, the survival of cells challenged with EGTA was assessed and the high sensitivity of the *pmr1Δ* strain was re-established to values identical to the wild type by GST overexpression (Fig. 4B).

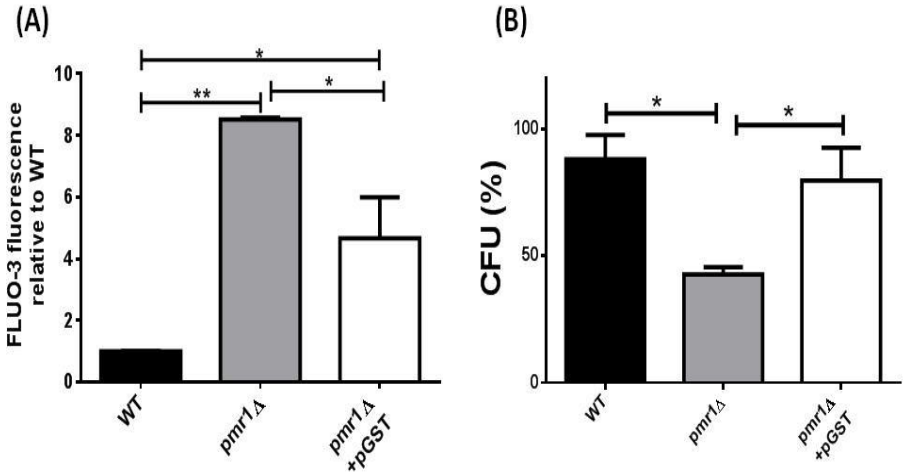

Fig. 4 (A) Intracellular calcium content measured by using FLUO-3AM probe and expressed as fluorescence relative to wild type strain. **(B)** Cell survival of the indicated yeast strains challenged with the calcium chelator EGTA for 6h. After incubation, cells were diluted and plated onto YPD plates. CFU were counted after growth at 28°C for 2 d and results were expressed as cell survival percentage in comparison to untreated controls, set as 100%.

1.4. The cell wall alterations in *Klpmr1Δ* cells are recovered by GST overespression

We previously reported that *K. lactis* strain deleted for *PMR1* gene had a thicker and disorganized cell wall (Uccelletti et al., 2005). We took advantage of the Calcofluor white (CFW), a fluorescent dye that binds primarily to chitin, one of the cell wall components. To analyze the effect of GST overexpression on cell wall structure, the CFW fluorescence assay was performed. After CFW staining, wild type cells showed the characteristic chitin deposition, localized only at the bud scars meanwhile the mutant strain exhibited an abnormal distribution of chitin across the entire cell surface. The overexpression of mammalian GTS completely suppressed the cell wall alterations of mutant cells. Indeed, like wild type cells, in the transformant strain the fluorescence is mainly localized at the division septum (Fig. 5).

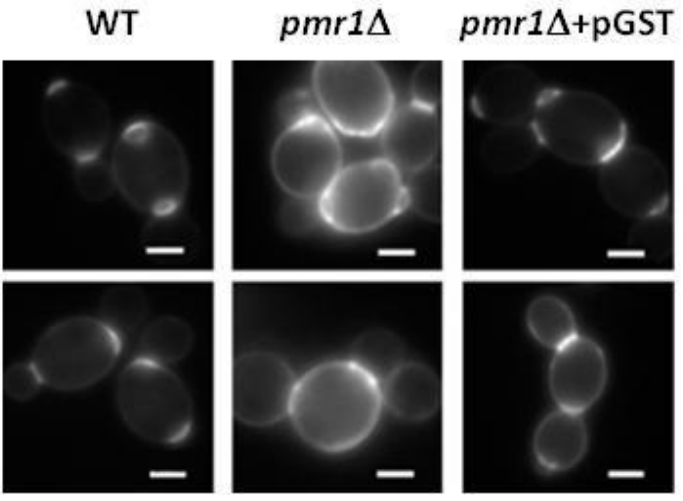

Fig. 5 Chitin distribution was analyzed by calcofluor white staining for the indicated strains grown to late exponential phase. All the pictures were taken at the same magnification; scale bar 2 μm.

1.5. GST effects on calcium homeostasis, cell wall and oxidative stress are mediated by calcineurin

Calcineurin (CN) is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase that is highly conserved in eukaryotes from yeast to mammals. It plays a crucial role in the Ca^{2+} signaling pathway (Miyakawa and Mizunuma, 2007) and its activation is necessary to maintain growth under different cellular stresses. In yeast, calcineurin is a heterodimer composed by a catalytic subunit (Cna1) and a Ca^{2+}-binding regulatory B subunit (Cnb1), and the association between the two subunits is necessary for phosphatase activity. Interestingly, the two-subunit structure is well conserved from yeast to human.

Thus, we explored if the phenotypes observed in *pmr1Δ* strain may be associated with defects in the calcineurin pathway. We analyzed the transcriptional levels of *CNA1* in wild type and mutant cells, and found no significant differences in *CNA1* transcriptional levels (Fig. 6A). However, the activation of calcineurin was evaluated by analyzing the expression of one of its target genes, *PMC1*. This gene encodes a vacuolar Ca^{2+} pump that works to sequester calcium inside the vacuole, the main calcium store of yeast cells. As shown in Fig. 6B, in *pmr1Δ* strain we found a high amount of *PMC1* transcript compared to wild type levels, indicative of CN activation. In fact, the increased levels of *PMC1* transcript were not observed when *CNB1* gene was deleted in those cells (Fig. 6B). These results strongly suggest that CN regulates the expression of *PMC1* gene in response to increased Ca^{2+} in the mutant cells. According to the high Ca^{2+} levels in mutant strain, the GST overexpression in *pmr1Δ* cells induced a partial reduction of *PMC1* transcript (Fig. 6B). Instead, no significant changes were observed for the *VCX1* gene encoding the vacuolar Ca^{2+} exchanger (Fig. 6C)

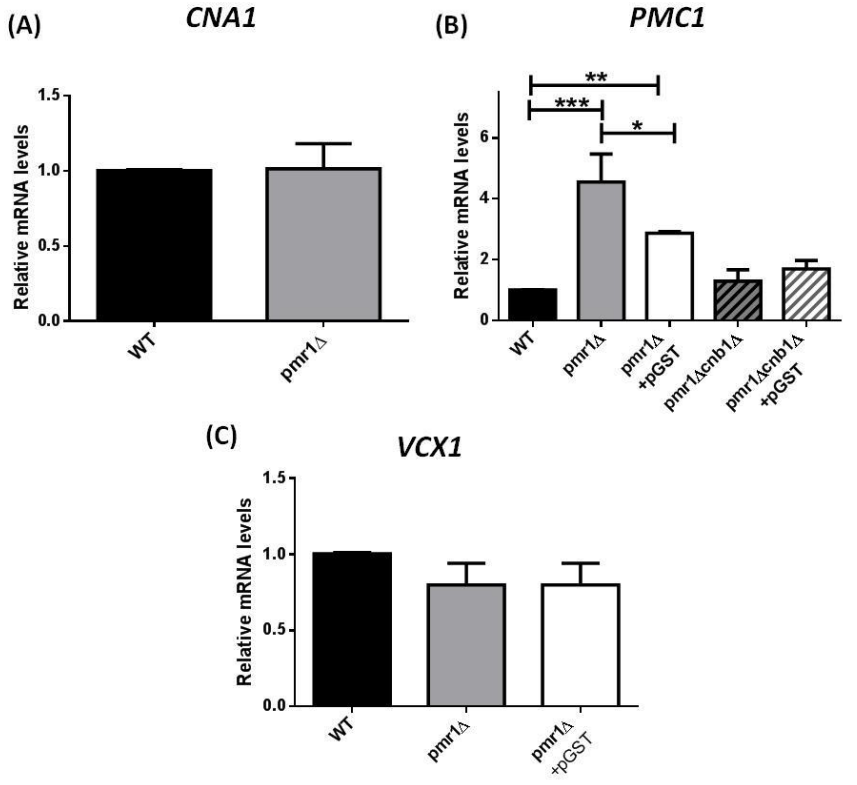

Fig. 6 Gene expression analysis of the regolaroty subunit of calcineurin *CNA1***(A)**, the vacuolar Ca^{2+} pump *PMC1* **(B)** and the vacuolar Ca^{2+}/H^{+} exchenger *VCX1***(C).**

Additionally, the calcium homeostasis defects, as well as the oxidative stress, observed in the mutant cells, were exacerbated in the *pmr1Δcnb1Δ* double mutant strain, as indicated by the higher sensitivity of this strain to the EGTA and H_2O_2 (Fig. 7A-B).
Moreover, calcineurin is a key element in the Cell Wall Integrity (CWI) pathway that is mainly activated by cell wall stress (Fuchs and Mylonakis, 2009). Since our mutant presented alterations in cell wall organization, we analyzed the GST effect on the cell wall in *pmr1Δcnb1Δ* double mutant strain. Our data revealed the inability of

GST to suppress also the cell wall morphology in the double mutant. (Fig. 7C).

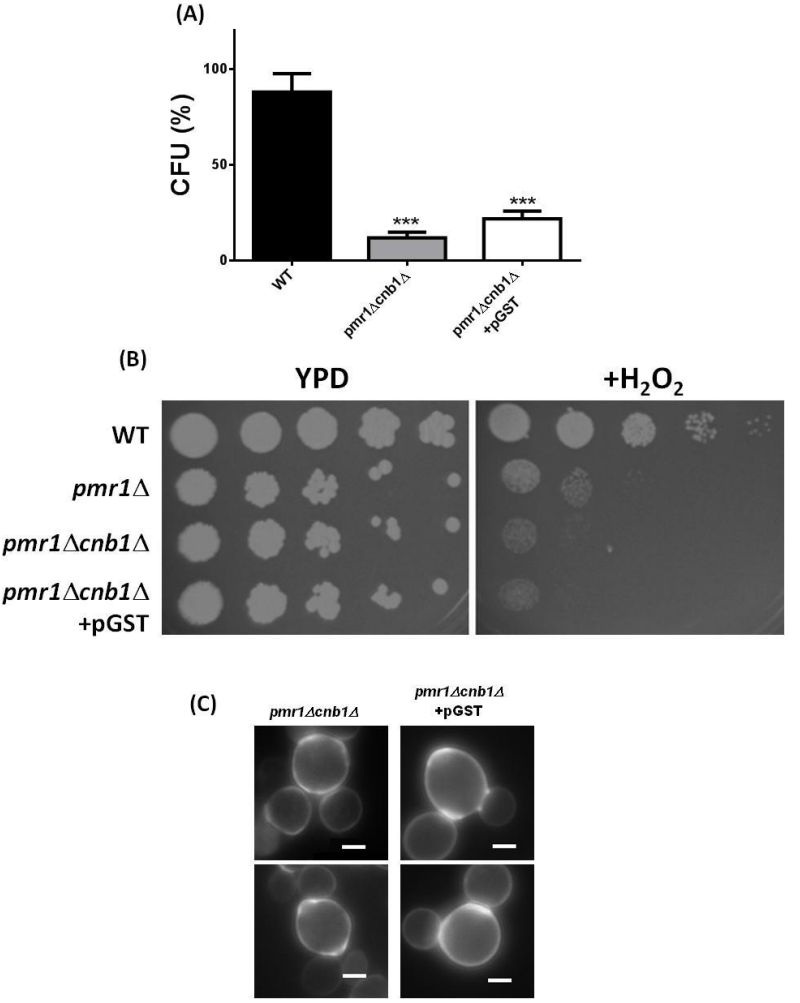

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Fig. 7 (A) Cell survival of the *pmr1Δcnb1Δ* strain, over-expressing or not GST,. challenged with the calcium chelator EGTA for 6h. **(B)** Sensitivity to hydrogen peroxide of the double mutant. **(C)** Cell wall analysis of the indicated strains through calcofluor staining of chitin. Scale bar 2 μm.

1.6. GST recovers the mitochondria functionality in a calcineurin-independent manner

Next we analyzed whether mammalian Glutathione S-Transferase was able to restore the mitochondria functionality when *CNB1* gene is inactivated in *PMR1*-deleted cells. Surprisingly, however, we found that GST overexpression was sufficient to restore mitochondrial functionality in the double mutants (Fig. 8), indicating in this case a mechanism of action for GST calcineurin independent.

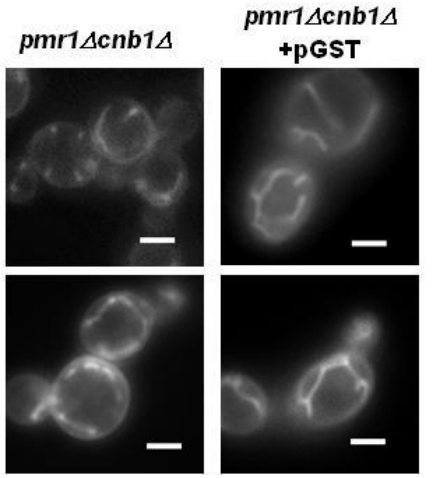

Fig. 8 DASPMI staining of the double mutant *pmr1*Δ*cnb1*Δ expressing or not the mammalian GST. All pictures were taken at the same magnification; scale bar 2 μm.

1.7. Alcohol dehydrogenase 3 recovers the mitochondria alterations of *pmr1Δ* cells

Since GST was able to suppress the mitochondrial defects of *pmr1Δ* cells calcineurin-independently, we tried to identify the possible mediator that allowed the mammalian protein to recover this phenotype.

We thus investigated the role of alcohol dehydrogenases (Adhs) that have a central role in the oxidative metabolism. These enzymes catalyze the production and assimilation of alcohols and they are ubiquitously distributed in most organisms (Lertwattanasakul et al., 2015). In *K. lactis* there are four isoforms of Adhs: Adh1 and Adh2 are cytosolic enzymes, meanwhile Adh3 and Adh4 are mitochondrial proteins (Saliola et al., 1990; Saliola et al., 1991). We investigated the activity of the KlAdhs in our strains; we found that, in the *pmr1Δ* cell over-expressing GST, the activity of the Adh3 was higher when compared to wild type and mutant cells (Fig.9).

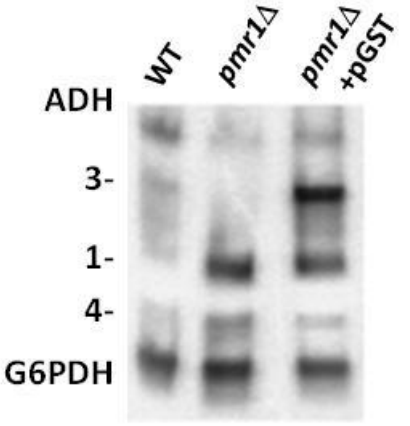

Fig. 9 Electrophoretic analysis of ADHs. 10 μg of protein, were electrophoresed on non-denaturing polyacrylamide gels and the ADH isozymes were revealed as bands by staining the gel for the enzyme activity. G6PDH activity was used as control.

KlAdh3 is responsible for maintaining the mitochondrial redox balance by contributing to the reoxidation of cytosolic NAD(P)H (Bozzi et al., 1997).
Thus, we decided to investigate directly the role of *KlADH3* in our mutant. As shown in Fig.10, the mitochondrial potential as well as the sensitivity to menadione, a generator of ROS in the mitochondrial compartment, were rescued in *pmr1Δ* cells by the increased expression of *ADH3*. However, both the calcium alterations (sensitivity to EGTA) and cell wall defects were not suppressed (Fig. 10).

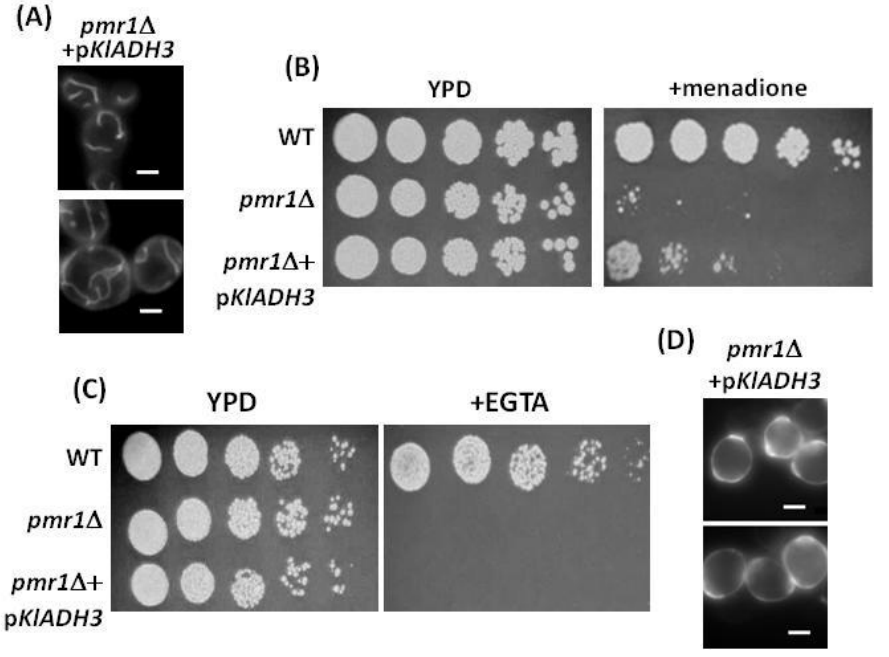

Fig. 10 Phenotypes of *pmr1Δ* cells overexpressing *KlADH3* gene. **(A)** analysis of the mitochondrial functionality by DASPMI; **(B)** sensitivity to the mitochondrial ROS generator, 60 μM menadione; **(C)** the indicated strains spotted on YPD plates with or without the calcium chelator, 20mM EGTA; **(D)** chitin staining with the fluorescent probe CFW. Scale bar for the fluorescent images: 2μm.

These results suggest that Adh3 has an important role in the mitochondrial redox homeostasis of Pmr1-depleted cells. Moreover, we can speculate that this enzyme could be a good candidate as GST mediator for the recovery of mitochondria defects and further analysis will be performed.

1.8. As in *Klpmr1* cells, GST genes are down-modulated in HHD keratinocytes

Based on these findings, we extended our study to HHD-derived keratinocytes. Thus, we analyzed whether also in HHD cells, like in yeast, the loss of *pmr1*/ATP2C1 induces a down-regulation of Glutatione-S Transferase. As shown in Fig. 11 A-B, our analysis indicated a decreased expression of GST-T1 and GST-M1 genes in HHD lesion-derived keratinocytes compared to non-lesion-derived keratinocytes from the same patients (Fig. 11 A-B). GSTs have been demonstrated to be important in protecting cells from oxidative stress by detoxifying some of the secondary ROS produced when primary ROS react with cellular constituents. Additionally, GSTs are transcriptionally controlled by NRF2, a transcription factor that regulates the expression of many cytoprotective genes implicated in the cellular response against oxidative stress. Our results showed that Nrf2 is down modulated in HHD derived cells (Fig. 11C); thus, indicating a mechanism through which GST down-modulation might be established in HHD-keratinocytes.

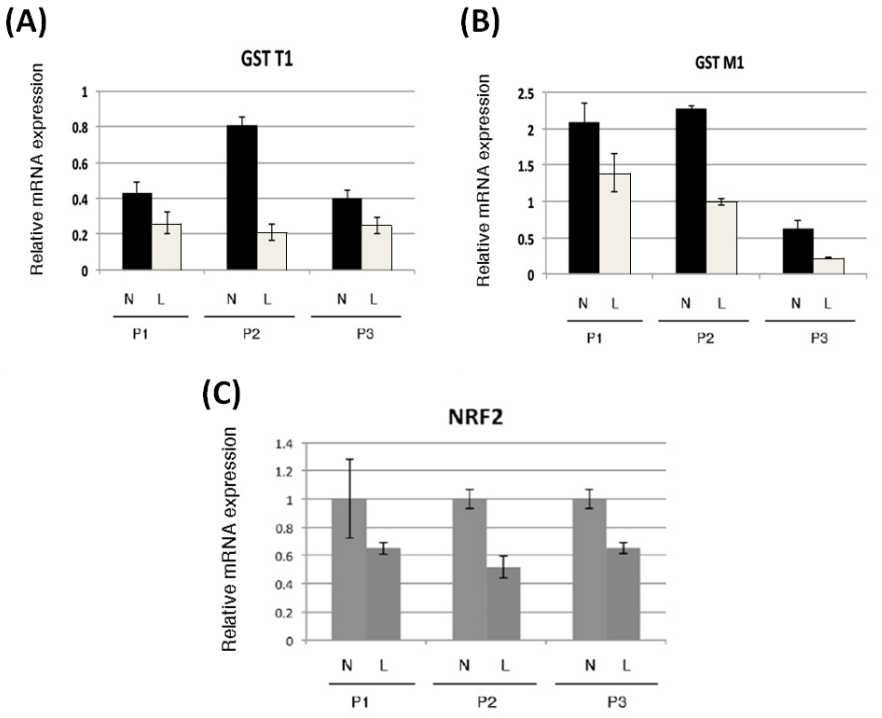

Fig. 11 Real-time PCR analysis of gene expression for GST-T1 **(A)**, GST-M1 **(B)** and NRF2 **(C)** in non-lesional (N) and lesional-skin (L) of three HHD patients (P1-P2-P3).

2. A yeast-based drug screening to identify therapeutic compounds for HHD

As mentioned before, currently there is not a specific treatment for Hailey Hailey disease. To identify compounds that could prevent the HHD manifestation, we initiated a screen for molecules that are able to rescue the main phenotypes of the *klpmr1*Δ mutant. We reasoned that the identification of positive hits would provide a primary set of compounds that can be further tested on human cell culture models of Hailey–Hailey disease.

The initial screening was performed using a set of 131 FDA-approved drugs and, for the sake of simplicity, we started evaluating their ability to alleviate the cell wall defects of the *klpmr1*Δ mutant.

To this aim, the CFW staining was performed on cells grown 24h in the presence of the different FDA-approved compounds at the concentration of 200 μM. The compounds that had partial or not effects were tested at the concentration of 250 μM. Only the drug S1973 (Cyclocytidine) had a bigger effect at 250 μM than 200μM, meanwhile the other compounds showed the same phenotype both at 200 and 250 μM. Furthermore, the molecules resulted toxic to 200 μM were retested at lower concentration (100, 10 and 5 μM) but they were discarded because still toxic or ineffective.

At the end of the screening, among the 131 compounds, 6 molecules showed at least 40% recovery of cell wall defects of mutant cells. Particularly, the compound S2386 strongly recovered the cell wall morphology in the 80% of *klpmr1*Δ cells (Fig. 12A-B).

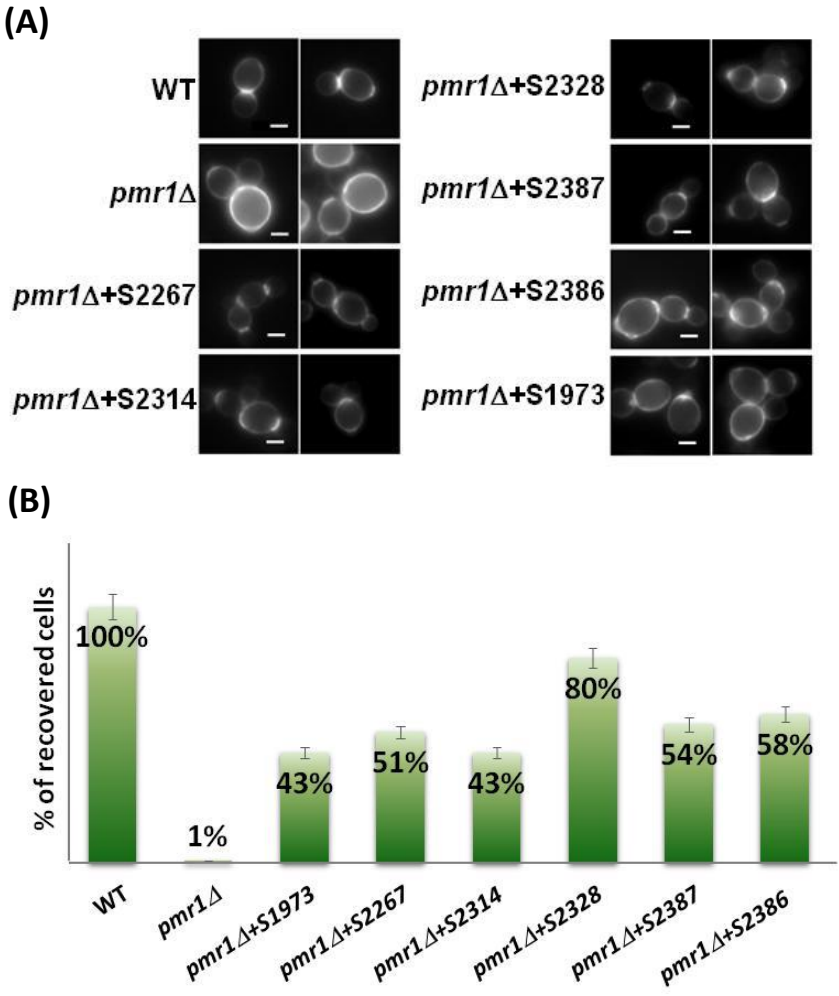

Fig. 12 (A) Pharmacological screening for the recovery of cell wall defects of *pmr1Δ* cells. Mutant strain were grown with individual compounds for 24h and then stained with CFW. The six positive hits are shown with wild type cells used as positive control. Scale bar 2 μm. **(B)** Percentage of cells with a WT-like chitin distribution. At least 200 cells per sample were scored manually as CFW-recovered or not.

The six molecules, selected from the preliminary screen, belong to different class of drugs (Fig. 13). Indeed, the S2386 and S1973 (Indirubin and Cyclocytidine) are used in medicine as chemioterapic

(Damiens et al., 2001; Novotný et al., 1990), the S2387 and S2314 (Lappaconite Hydrobromide and Kaempferol) have an anti-inflammatory action (Murota *et al*., 2002), and S2328 and S2267 (Nalidixic acid and Azomycin) are mainly recognized as anti-bacterial drugs (Bourgnignon et al., 1973; Saeki et al., 1974).

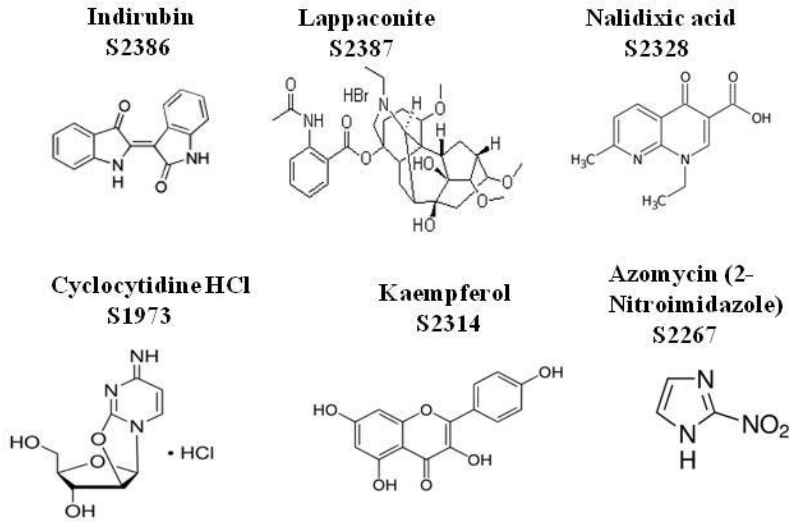

Fig. 13 Chemical structures of the six natural compounds selected in our pharmacological screening

Our next goal was to assess if these six molecules were also able to rescue other defects of the mutant cells, as for example the calcium alterations. For this aim, we tested the sensitivity of the *klpmr1Δ* cells treated with the different drugs to EGTA (Fig.14). In this case two compounds had positive effect: S2386 (Indirubin) and S1973 (Cyclocytidine), meanwhile the other tree molecules had not effects.

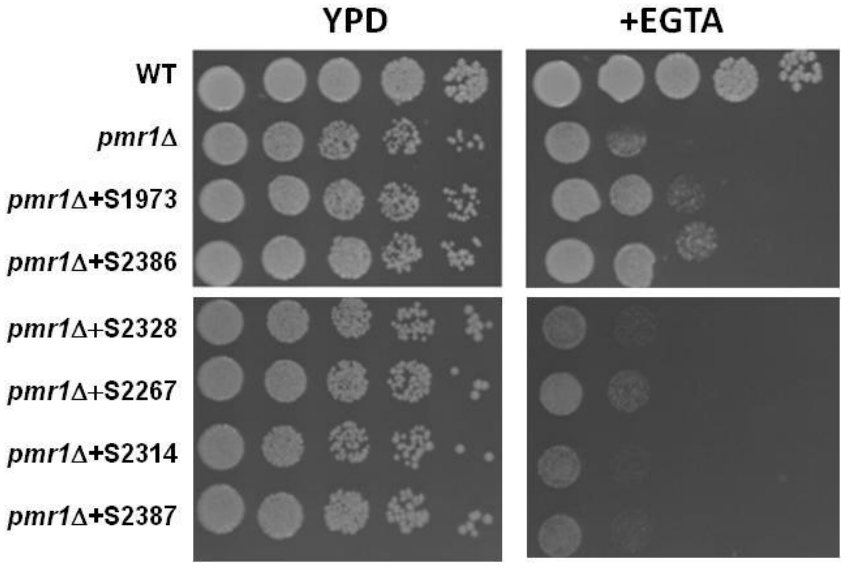

Fig. 14 Growth of WT and *pmr1Δ* cells treated or not with the natural compounds onto solid medium supplemented with 20 mM EGTA.

Since the lack of *PMR1* in yeast as well as in human HHD keratinocytes, induced an increase of ROS production (Cialfi et al., 2010; Uccelletti et al., 2005), we investigated if the positive hits of the primary screen had effects also on the oxidative stress that characterized our mutant. The two ROS generators, menadione and H_2O_2 were used to this aim (Fig. 15). While menadione is an endogenous generator of ROS, H_2O_2 is an exogenous one. As shown in figure 15 the molecules 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 resulted more effective against H_2O_2. This indicates that the action of the different compounds depends on the localization of the ROS source.

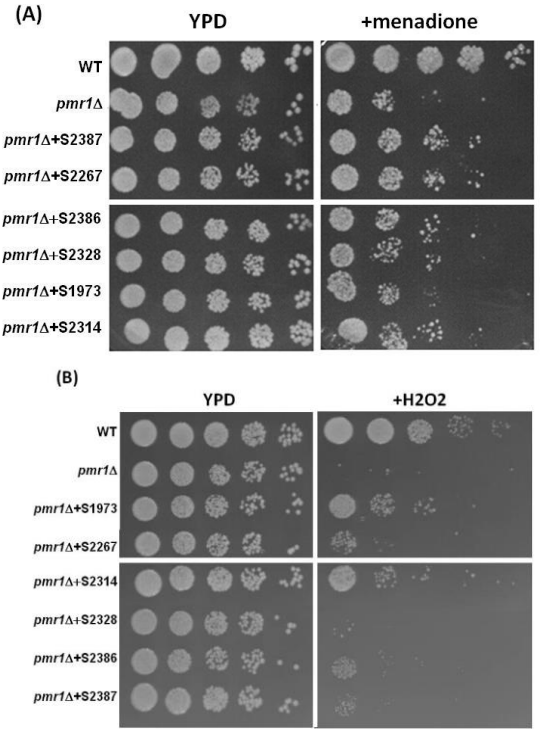

Fig. 15 Sensitivity of *PMR1*-deleted cells, grown with or without the single compound, to **(A)** 60μM menadione or **(B)** 4 mM H_2O_2.

Mitochondria are responsible for the main source of ROS in most cells, linking mitochondrial respiration with ROS effects on cellular function (Murphy, 2009). As showed before, *klpmr1*Δ cells have an impaired mitochondrial functionality with a spotted network compared to a wild type tubular one. For this reason, we evaluated if our selected compounds could restore the mitochondrial functionality in the mutant strain, using the fluorescent probe DASPMI (Fig 16). We found that 3 drugs (S2386, S2314 and S2387) restored the tubular mitochondrial network of wild type cells.

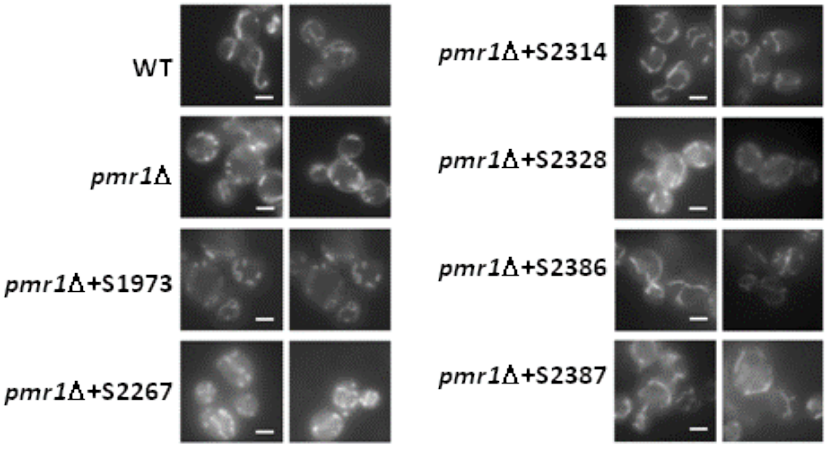

Fig. 16 Assessment of the mitochondrial functionality for *pmr1Δ* cells untreated or treated with the indicated drugs. WT strain was used as a control. Scale bar 2 μm.

In conclusion, the data obtained showed that although all the six drugs alleviate the cell wall defects of the mutant strain, each of them acts on different phenotypes of *klpmr1*Δ cells. This can agree with the fact that the selected molecules belong to different class of drugs (Fig. 13).
However, the molecular mechanisms by which these compounds improve cell survival of the mutant cells still need to be deciphered. Moreover, these drugs now need to be validated on human cell-based models of Hailey–Hailey disease.

DISCUSSION

Yeast is largely used as simple model organism to investigate the molecular players associated with the human diseases. This happens because the biological properties of the yeast like rapid growth, the ease of mutant isolation, a well-defined genetic system, and most important the fact that about 30% of the genes known to be involved in human diseases have a yeast orthologue (Botstein et al., 1997; Foury, 1997).

The recognition that the human orthologue of *PMR1* is affected in Hailey-Hailey *disease inspired yeast researchers to utilize this powerful model* organism in exploring the pathophysiology and potential therapy of this skin disease.

In *S. cerevisiae* it has been reported that *PMR1*-defective cells are sensitive to both low and high levels of calcium in the extracellular environment (Kellermayer, 2005). The mutant strain has elevated levels of cytosolic and total cellular calcium as well as susceptibility to manganese and oxidative stress (Halachmi et al., 1996; Lapinskas et al., 1995). Due to a perturbed ER and Golgi Ca^{2+}/Mn^{2+} homeostasis, *pmr1Δ* cells are also defective for protein processing through the secretory pathway and are sensitive to ER stress (Park et al., 2001). Thus, much of what we know about Hailey-Hailey disease originally came from studies in *S. cerevisiae*.

Currently, more than 100 mutations in human ATP2C1 suspected to be pathogenic in Hailey-Hailey disease have been identified. One obstacle that researchers have approaching the HHD-biology is the difficulty in obtaining skin samples due to the rarity of HHD and lack of an animal model. Moreover, there is no a relevant mammalian Hailey-Hailey disease model. Indeed, homozygous mutant mice exhibited no manifestations of HHD but did develop squamous cell carcinomas and papillomas (Okunade et al., 2007), while occurrences of squamous cell tumors is very rare in HHD patients.

In this study we used the yeast *Kluyveromyces lactis* as a model to find out the molecular basis of HHD. Like *S. cerevisiae, K. lactis* is genetically tractable and the components of human calcium storage/handling is conserved. However, *K.lactis* is a Crabtree-negative and obligate aerobe organism that responds to changes in oxygen availability like mammalian cells.

Based on this similarity between human and yeast calcium handling, we performed a genetic screening on *K.lactis pmr1Δ* cells, with a MDCK cDNA library, to isolate possible molecular interactors of *PMR1*/ATP2C1.

This allowed the identification of GST as a candidate gene associated with Hailey-Hailey disease manifestation. Indeed, from the genetic screening we selected one positive clone that was able to grow in the presence of hydrogen peroxide. After the sequencing, we found that the cDNA encoded the Glutathione S-transferase belonging to the Tetha class. Glutathione S-transferases (GSTs) are important in the detoxification of many compounds, including reactive oxygen species. The reduced and oxidized forms of glutathione (GSH and GSSG) act in concert with other redox-active compounds (e.g., NAD(P)H) to regulate and maintain cellular redox status. GSH, which is the main antioxidant in yeast (Lushchak, 2012), is used by GSTs to protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics, and thereby defend cells against the mutagenic, carcinogenic, and toxic effects of the compounds.

We found that in the *pmr1Δ* mutant, GST expression is decreased compared to WT and this would render this strain less tolerant to cellular stress and consequently would influence cell viability. Increased levels of ROS is a hallmark of both keratinocytes derived from HHD lesions and yeast *pmr1Δ* cells. The results that we obtained further demonstrate the importance of oxidative stress for *pmr1Δ* phenotypes and indicate the role for GST in protecting cells against oxidative damage; it can be inferred that ROS detoxification represents its main effect.

Moreover, we can speculate that GST might affect the redox status of Calcineurin and increase its anti oxidative stress function. It has been described that oxidative stress is a very potent CN inhibitor, acting by altering the redox state of iron at the CN active site. Interestingly, CN activity decreased in cells that were treated with L-buthionine-(S,R)-sulfoximine, an inhibitor of synthesis of the antioxidant glutathione (Alba et al., 2012).

In this context, decreased GST activity in *PMR1*-deleted strain would influence cell response to oxidative stress.

Furthermore, here we demonstrate the importance of CN-dependent GST activity for protection against oxidative stress. Indeed, we found

that *pmr1Δcnb1Δ* double mutant was hypersensitive to exogenous stress, and GST overexpression was unable to restore cytosolic Ca^{2+} levels as well as tolerance to exposure to either H_2O_2 or EGTA and cell wall defects. Ca^{2+} overload as well as oxidative stress might lead to altered mitochondrial morphology through a CN-dependent mechanism. However, after overexpression of GST in *pmr1Δcnb1Δ* double mutant mitochondrial tubular morphology is restored. The suppression of mitochondrial phenotype indicates that GST exerts a direct effect on the mitochondria morphology independently of CN signaling.

Our data, demonstrating that GST-mediated activity is an essential mechanism for maintenance of the mitochondrial functionality, is supported by the finding that altered GST activity results in increased mitochondrial stress and increased ROS production (Zhang et al., 2012).

Moreover, preliminary data suggest that the metabolic enzyme Adh3 could help the glutathione S-transferase in the recovery of the mitochondrial defects of *pmr1Δ* cells. Indeed we found that the activity of this alcohol dehydrogenase was particularly higher in the mutant cells overexpressing GST. Furthermore, this hypothesis was supported by the findings that the overexpression of *K. lactis ADH3,* in *PMR1*-deleted cells, restored the functionality of mitochondria and reduced the sensitivity to menadione, that selectively increases the ROS production at the mitochondrial level.

In order to validate the observations gathered from yeast, the analysis was extended to HHD-derived keratinocytes. Our findings showed that, as in *pmr1Δ* yeast mutant, HHD-lesion-derived keratinocytes were characterized by decreased expression of GST genes compared to non-lesion-derived keratinocytes from the same patients. The activities of GSTs, like other antioxidant enzymes, are controlled at transcriptional, translational, and posttranslational levels. In animals, the activities of many phase II detoxifying enzymes, including GSTs and Glutathione peroxidases (GPxs), are upregulated via the Nrf2/Keap1 system via interaction with antioxidant response elements (ARE) in the regulatory regions of many of these genes (Itoh et al., 1997; McMahon et al., 2001). Consistent with reduced GST-T1 and GST-M1 expression, we observed a decrease expression of the transcriptional factor NRF2 in HHD-lesion-derived keratinocytes.

In conclusion, the Glutathione S-transferase theta subunit was identified by a yeast genetic screening as a suppressor of the oxidative stress taking place in cells lacking the Golgi Ca^{2+}/Mn^{2+}−ATPase. In both yeast and HHD-derived keratinocytes, a down regulation of the corresponding GST genes was found, showing as *K.lactis* can be a model system for further investigation of the molecular mechanisms underlying this skin disorder.

To date, a real cure for HHD is absent and the treatment of HHD patients is mainly focalized on antifungal, antibiotic or antiviral to reduce the secondary infections (Burge, 1992; Le Saché-de Peufeilhoux et al., 2012) and corticosteroids to reduce inflammation (Ikeda et al., 1993). Other treatments aim to prevent the disease-triggering factors such as exposure to sunlight, friction or skin sweating. An example for the latter is the use of Botulinum toxin to reduce sweating and limiting the outbreak of HHD (Bessa et al., 2010). Reports of drug with positive effects on HHD lesions exist but they are mainly based on case studies. These reports include photodynamic treatment (Ruiz-Rodriguez et al., 2002) or the topical application of active compounds, like Aminoglycosides and vitamin D3 derivates (Clancy et al., 2001; Aoki et al., 1998), but a validated cure for all the patients is still faraway**.**
Following the large use of yeast as a powerful model for human diseases, it is widely recognized that yeast-based pharmacological screenings provide greater ease of genetic manipulation and it can be made rapidly at low cost.
This is more relevant in the case of HHD due to the lack of good animal models. Thus, we established a yeast-based screening assay designed to identify drugs that could be active against Hailey Hailey disorder.
For the sake of simplicity, in the initial screening, we tested 131 FDA-approved natural compounds for their ability to suppress the cell wall defects of the *pmr1Δ* mutant. This primary screen allowed the identification of six positive hits: Indirubin (S2386), Cyclocytidine (S1973), Nalidixic acid (S2328), Azomycin (S2267), Kaempferol (S2314) and Lappaconite Hydrobromide (S2387). As mentioned before, the six compounds belong to different classes of drugs. Indeed, Indirubin and Cyclocytidine are chemioterapics, Kaempferol and Lappaconite Hydrobromide have anti-inflammatory action, Azomycin

and Nalidixic acid are commonly used for their anti-bacterical action. This could explain because we observed that some compounds are active on different phenotype of our mutant.

In our screening, the less effective drug resulted the Nalidixic acid that is able to recover only the cell wall defect of *pmr1Δ* cells. This drugs is an anti-microbic agent described as a specific inhibitor of bacterial DNA synthesis in *vivo* and in *vitro* (Bourgnignon et al., 1973), besides it also prevents protein synthesis in eukaryotic organisms, such as *S. cerevisiae* (Whittaker and Carnevali 1977). It is reported that the ability of *S. cerevisiae* cells, treated with pulsed electric fields (PEF), to repair the membrane damage in the presence of Nalidixic acid would require, to some extent, nucleic acid or protein synthesis (Somolinos et al., 2008). Although the effects of Nalidixic acid on cell wall are unknown, we speculate that also in the recovery of cell wall defects of our mutant, Nalidixic acid could act on the nucleic acid or protein synthesis.

In the next step of the screening, we evaluated whether the six selected compounds were able to affect other phenotypes of *PMR1*-deleted cells. We found that the growth of *pmr1Δ* cells on Ca^{2+} chelator, EGTA, was recovered only by the two chemioterapic compounds Cyclocytidine and Indirubin.

Cyclocytidine is the prodrug of cytarabine, which is a pyrimidine nucleoside analog that inhibits the DNA synthesis and used mainly in the treatment of leukemia (Novotný et al., 1990).

It displayed selective growth inhibition against the tumor cell lines with *ras* oncogenes that is the most frequently occurring gain-of-function mutation detected in human tumors (Koo et al., 1996).

Instead, Indirurbin is a red colored 3,2-bisindole isomer and one of the component of *Indigo naturalis*, as well as an active ingredient used in traditional Chinese medicine for the treatment of chronic diseases. It selectively inhibits CDKs and other kinases like GSK-3 blocking cancer cell proliferation in the late G1 and G2/M phases of the cell cycle (Damiens et al., 2001).

Since *K. lactis* cells lacking of the Ca^{2+}/Mn^{2+}-ATPse present also alterations in the cell cycle (Uccelletti et al., 1999), probably Indirubin and Cyclocytidine act on determinate components of cell cycles thus, in some way, they restore the growth of mutant cells in the presence of EGTA.

We also investigated the ability of the selected drugs to alleviate the oxidative stress of *pmr1Δ* cells. To understand if the drugs effects could be influenced by the source of ROS, we used menadione and H_2O_2, generating ROS at mitochondrial and extracellular level respectively.
Indeed, Indirubin, Azomycin, Lappaconite Hydrobromide and Kaempferol acted on the mitochondrial-derived ROS, recovering the growth on menadione; meanwhile Cyclocytidine and Kaempferol allowed *PMR1*-deleted cells to grow in the presence of H_2O_2.
Azomycin is an antimicrobial antibiotic produced by a strain of *Nocardia mesenterica*. It was studied its biochemical effect mostly on *Bacillus subtilis* and *Escherichia coli* in which inhibits the activity of ribonucleotide reductase involved in the nucleic acid synthesis (Saeki et al., 1973).
Lappaconite Hydrobromide is instead a kind of alkaloid extracted from *Aconitum sinomontanum Nakai* and has anti-inflammatory effects. For both Azomycin and Lappaconite few is known about the mechanism of action and further analysis are necessary to understand their role in the oxidative stress response.
Very interesting the flavonoid, Kaempferol, recovered the growth on both menadione and H_2O_2, showing a broader spectrum of action agaist ROS. However, the antioxidant properties of Kaempferol are well know. Indeed, it exhibits strong radical-scavenging activity (Murota et al., 2002), inhibits formation of superoxide anion radicals by xanthine oxidase (Selloum et al., 2001) and reduces the damage to DNA induced by H_2O_2 (Noroozi et al., 1998). In the nematode *C. elegans* Kaempferol reduces the ROS boost induced by thermal stress and prolonged the survival at lethal temperature inducing the nuclear localization of the FoxO transcription factor, DAF-16 (Kampkötter et al., 2007). Moreover in human keratinocyte cell line, HaCaT, Kaempferol controls the transcription of several genes by the regulation of key transcriptional factor involved in the oxidative stress and inflammation like Nrf-2 and Nf- κB (Kang et al., 2008). Finally analyzing the mitochondrial functionality of *pmr1Δ* cells treated with the six drugs we found that while Indirubin and Lappaconite Hydrobromide partially recovered the $\Delta\Psi$ alteration of mutant strain, Kaempferol totally restored a mitochondria WT-like tubular network.
Nothing is reported about the role of Lappaconite Hydrobromide on mitochondrial membrane potential; while, Varela and colleagues

demonstrated that, in the isolated rat liver mitochondria, Indirubin derivative indirubin-3'-oxime impairs the oxidative phosphorylation but on the other hand, it had a partial preventive role on the mitochondrial permeability transition induced by calcium overload (Varela et al., 2008). In the same way in the *pmr1* mitochondria, Indirubin could partial recover the membrane potential alterations caused by the loss of the Ca^{2+} pump.

The protective role of Kaempferol on mitochondria is well reported in literature. Indeed Choi in 2011 demonstrated that pre-treatment with Kaempferol reduced the mitochondrial membrane potential dissipation as well as the ([Ca2+]i) elevation, and ROS production in osteoblast-like MC3T3-E1 cells treated with antimycin A (Choi, 2011). Furthermore, mitochondrial Ca^{2+} uptake is enhanced by treatments with Kaempferol (Montero et al., 2004; Kannurpatti et al., 2015) indicating a regulation of mitochondrial Ca^{2+} homeostasis by Kaempferol. Thus, in *PMR1*- deleted cells Kaempferol could restore the mitochondrial functionality by scavenging the ROS and controlling Ca^{2+} levels of the organelle.

In conclusion, our screening allowed the identification of six compounds able to alleviate one or more phenotypes of *pmr1Δ* cells. The most promising molecules resulted the indol Indirubin and the flavonoid Kaempferol.

However, further analysis are necessary to understand the mechanisms of action and the possible molecular targets of these compounds. Lastly, these drugs will be tested in human cell-based models of Hailey–Hailey disease to be validated.

WORK IN PROGRESS

1. *C. elegans*: a simple multicellular model to study HHD

As shown before, in HHD keratinocytes, the transcription factor Nrf2 is down modulated. In order to study the involvement of this factor in the phenotypes associated to the pathology, we decided to consider another simple organism, largely utilized for modeling human diseases: the nematode *Caenorhabditis elegans.* This choice was mainly due to the lack of NRF2 in yeast cells.
The roundworm *C. elegans* is a free-living, non-parasitic nematode, with a life cicle of 3.5 days at 20°C and a lifespan of about 2–3 weeks under suitable living conditions. Hatched animals develop through four larval stages (L1–L4) to arise as an adult hermaphrodite with 959 somatic cells (Sulston and Horvitz, 1977) and a body length of about 1 mm and 80 μm in diameter (Fig. 1).

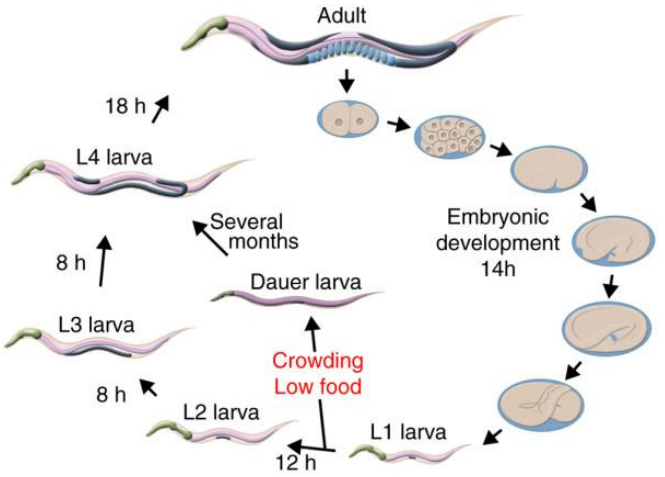

Fig. 1 *C. elegans* life cycle. Under favorable conditions animals pass through direct development to adulthood in as little as 3 to 4 days. In response to hard environmental conditions, like food shortage, crowding or high temperatures, animals can enter into an arrested dauer stage (Murgatroyd and Spengler, 2010).

This simple metazoan can be easily cultivated in large numbers and the transparent body permits to track cells and follow cell lineages and biological processes (Sulston et al., 1983). In 1998 the whole genome of *C. elegans* was sequenced and it has been estimated that about 42% of the human disease genes have an orthologue in the *C. elegans* genome.

At the beginning, a RNAi clone to inactivate *pmr-1* was constructed (see materials and methods) and the effects of *pmr-1* inactivation was evaluated in terms of EGTA sensitivity. To this aim, a lifespan assay was performed using L4 one day-adult animals interfered for 48h with *pmr-1*(RNAi) or with *E. coli* transformed with the empty plasmid, *EV* (RNAi), and then supplemented continuously with 20mM EGTA. As shown in the Fig 2, the EGTA treatment induced a strong reduction of the survival rate of *pmr-1* worms when compared to the control animals (Fig. 2).

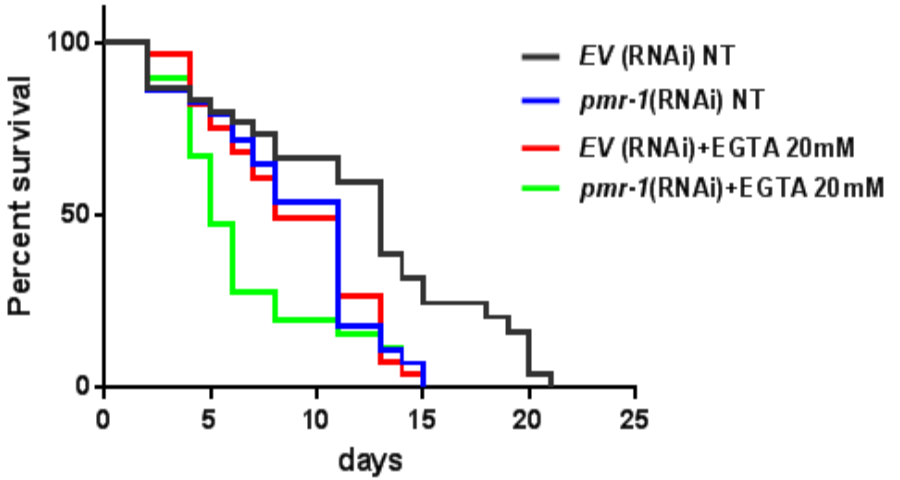

Fig. 2 Lifespan analysis of N2 animals fed with *EV*(RNAi) or *pmr-1*(RNAi). L4 one day-adult animals, after 48h of interference, were shifted on RNAi plates supplied with 20mM EGTA (To). From this point, the worms were interfered and treated with EGTA continuously during the life span. The untreated animals (NT) were used as a control. n=50 for each data point of single experiment.

Moreover, we investigated if the Nrf2-homolog gene, *skn-1*, was down-modulated in PMR-1 depleted worms, as for the mammalian counterpart in HHD keratinocytes. For this aim, we used a transgenic strain (LD1) homozygous for a reporter gene (ldls7), which consists of a fusion of the *skn-1b/c* promoter to GFP (*skn1*::GFP).
Transgenic one day-adult worms were fed with *EV (*RNAi) or *pmr-1* (RNAi) for 48h or 72h and then the fluorescence was analyzed. The results obtained indicated that, also in *C. elegans*, the transcriptional factor NRF2/SKN-1 is down-modulated; indeed the GFP fluorescence was lower in the *pmr-1* worms when compared to the control (Fig. 3).

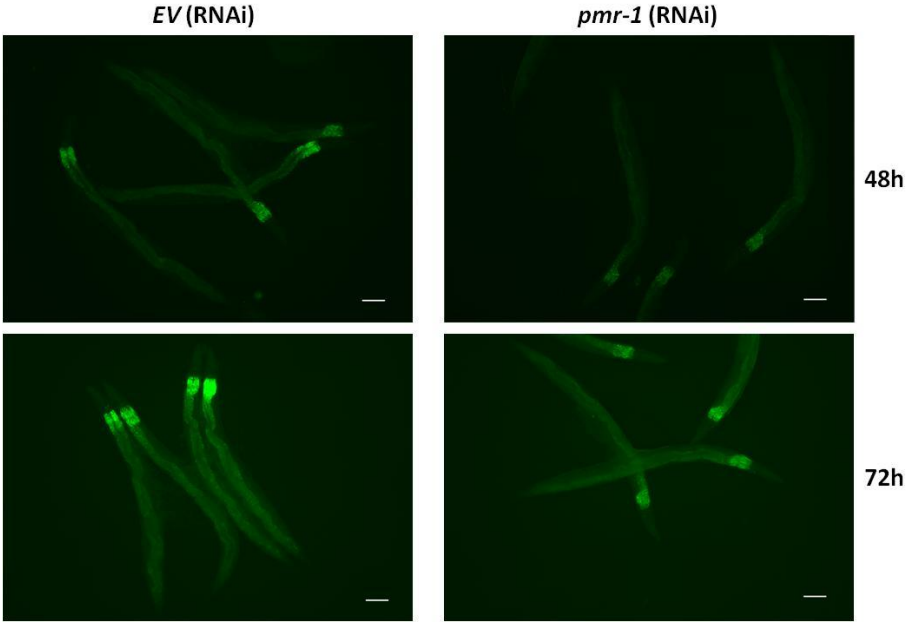

Fig. 3 *skn-1*::GFP expression in LD1 worms treated with *EV*(RNAi) or *pmr-1*(RNAi) for 48h or 72h. The pictures were taken at the X5 magnification; scale bar 100μM

Thus, when ATP2C1/PMR-1 is not expressed, the down-modulation of NRF2/SKN-1 occurs in both HHD keratinocytes and the nematode *C. elegans*.

However, these preliminary data need to be widened by further experiments. We are currently investigating the role of key players involved in the pathways those cross-talk with SKN-1, such as the insulin/IGF-1 receptor, DAF-2, and the FOXO transcription factor, DAF-16 (Fig.4).

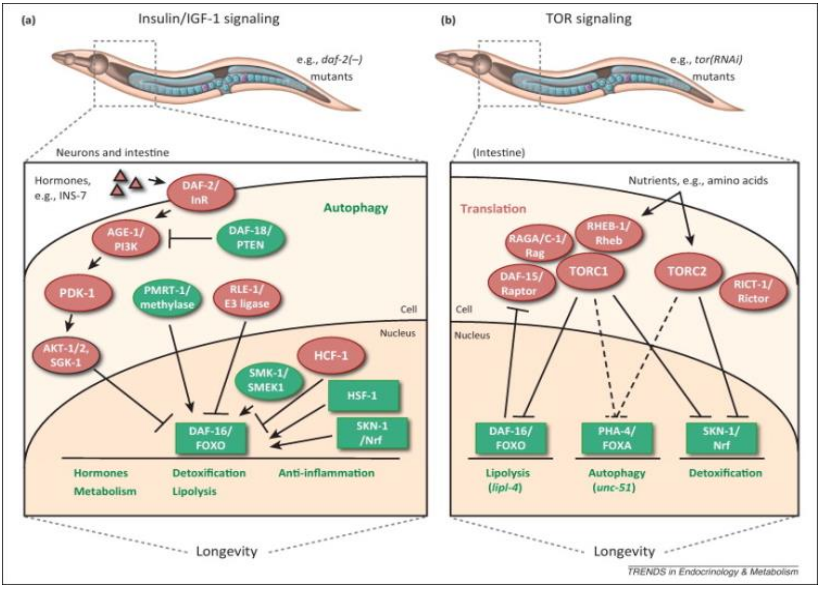

Fig. 4 Molecular interaction of SKN-1 with the DAF-2 and DAF-16 pathways (Lapierre and Hansen, 2012).

MATHERIALS AND METHODS

1. Yeast strains and 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 centromeric Kcplac13 plasmid harboring the *KlADH3* ORF (kindly provided by Dr. Michele Saliola) was used to overexpress the *K. lactis* Alcohol dehydrogenase 3 in the *pmr1Δ* cells.

The double mutant *pmr1Δcnb1Δ* was obtained by transforming the CPK1 strain with the disruption cassette for *CNB1*. The deletion cassette was obtained by cloning the 5' and 3' regions of *CNB1*, amplified with the following primers (5'*CnbF* 5' TGTTCAACCACGAGCATAAAA-3'; 5'*CnbR* 5'-CCTCTTCCACTGAAGATGGAT-3'; 3'*CnbF* 5'-CGGTTACATATCCAATGGTGA-3'; 3'*CnbR* 5'-CGTCAATGCCAGAGAAAACA-3'), in the TA vector (Invitrogen). Afterward these fragments were subcloned in pKSURA3 carrying the URA3 gene of *S. cerevisiae*. After amplification of the disruption cassette with the above mentioned 5'*CnbF* and 3'*CnbR* primers, the MW278-20C strain was transformed and the transformants were selected on synthetic complete (SC) media without uracil and then analyzed by PCR to test the correct integration of the cassette.

Yeast strains were grown in SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids) with the appropriate auxotrophic requirements. Five-fold serial dilution from concentrated suspensions of exponentially growing cells (5×10^6 cell/ml) were spotted onto synthetic YPD agar plates (1% yeast extract, 1% peptone, 2% glucose, 2% agar) supplemented or not with 4 mM H_2O_2. The plates were incubated at 30°C for 3 days.

2. Screening of MDCK cDNA library

The CPK1 strain was transformed to saturation with the MDCK cDNA library constructed in the
pEPGK31 multicopy vector (kindly provided by Dr. Claudia Abeijon) by electroporation. All the Ura^+ transformants were replica plated on to YPD medium supplemented with 4 mM H_2O_2.
The plasmids isolated from the Ura^+/ H_2O_2 transformants were used to transform the *pmr1Δ* strain. Plasmids capable of restoring the H_2O_2 phenotype to the *pmr1Δ* after retransformation were analyzed. Molecular analysis by restriction enzymes of the DNA fragments from the isolated plasmids showed that one of these plasmids carrying a fragment of about 800 base pairs (bp) was able to restore the H_2O_2 R phenotype and then was sequenced (BMR Genomics). Afterward, the fragment resulted to be the cDNA of the GST theta class and it was subcloned into the multicopy
vector pCXJ6-L (Zanni et al., 2009) to obtain the pGST plasmid. Yeast strains were then transformed with pGST plasmid or the empty vector pCXJ6-L, used as a control. In the case of *GTT1* expression, the *K. lactis* gene was cloned in p426SD11 vector by amplifying the ORF with the following primers: GTT1orf_F 5'–CGGGATCCTCAGATACACGCATCCCAC-3' and GTT1orf_R 5'–CTCGAGGAATGAGGCGCCATTCGCTT-3' (the BamHI and XhoI restriction sites are single and double underlined, respectively). The PCR product was ligated into the pGEM-T-Easy vector (Promega) and after sequencing (MWG Biotech, Ebersberg, Germany) the fragment was cloned in BamHI/XhoI-digested p426SD11 plasmid, obtaining pGTT1 vector.

3. Fluorescence microscopy

Late exponential-phase cells grown in SD minimal medium were harvested, washed with water and then incubated with the vital dye 2-(4-dimethylaminostyryl)-N-methylpyridinium iodide (DASPMI; Sigma) by the method of (Guthrie and Fink, 2002). Chitin staining was

performed by the method of (Uccelletti et al., 1999). Epifluorescence microscopy was carried out with a Zeiss AxioVert 25 microscope fitted with a ×100 immersion objective and a standard filter set.

4. Cytofluorimetric analysis

To evaluate the ROS accumulation, 10^8 cells were harvested and washed twice in PBS and then were incubated in PBS at 28°C with Dihydroethidium (DHE) for 10 min. After the incubation, the cells were washed three times in PBS and then were analyzed. To measure the intracellular Ca^{2+} levels, 10^8 yeast cells, harvested and washed twice in PBS, were incubated in PBS at 37°C for 30 min with 10 μM Fluo-3-acetoxymethyl ester (Fluo-3/AM; LifeTechnologies). A non-cytotoxic detergent, pluronic F-127 (0.1%), was added to increase solubility of the Fluo-3/AM. Then yeast cells were washed twice in PBS and analyzed. Fluorescence was measured by Becton-Dickinson FACSCalibur flow cytometer.

5. Quantitative Real-time PCR from yeast cells

RNAs were extracted through the hot phenol method as described in (Zanni et al., 2015) and then digested with 2U/μl DNAse I (Ambion). A total of 1 μg of each sample were reverse-transcribed using oligo dT and SensiFAST cDNA Synthesis Kit (Bioline), according to the manufacturer's instructions. The cDNA than was diluted at final concentration of 20 ng/μl. Real-time PCR was performed using SensiMix SYBR (Bioline) and selective primers (200 nM) designed with Primer3 software (Table S1). Each well contained 2μl of cDNA used as template and all samples were run in triplicate. An I Cycler IQ™ Multicolor Real-Time Detection System (Biorad) was used for the analysis. Real-time PCR conditions included a denaturing step at 95°C for 3min, followed by 40 cycles at 95°C for 30s, 55°C for 30s and

75°C for 45s. Two cycles were included as final steps: one at 95°C (1 min) and the other at the annealing temperature specific for each couple of primers used (1 min).
Quantification was performed using a comparative CT method (CT=threshold cycle value). Briefly, differences between the mean CT value of each sample and the CT value of the housekeeping gene (ACT1) were calculated: ΔCTsample=CTsample−CT ACT1.
The final result was determined as $2^{-\Delta\Delta CT}$ where ΔΔCT=ΔCTsample−ΔCTcontrol.

Table 1S: Primer sequences

Gene name	Forward (5'to3')	Reverse (5'to3')
KlACT1	AATGCAAACTGCTTCTCAAT	AACAGATGGATGGAACAAAG
KlCNA1	GTTAATGCAGCTCTG	CACGTGATAGTCGTC
KlCNB1	TGTTCAACCACGAGCATAAAA	CGTCAATGCCAGAGAAAACA
KlCTA1	CCTCATAAGCAGTTCCCATT	CTAATCTGTAACGATGGGCA
KlCTT1	TATTCTCGTACCCAGACACT	TTGGTCCTTGAAGTTGAACA
KlGTT1	GCTTCAACCACCTTTATTCA	CGGTCTCAATGAAATCTAGC
KlPMC1	ATCAAGTCTGCTGTCGTTTT	GATAAGGGTTTTCATCCACA
KlSOD1	ATGCTTTGAGAGGTTTCCAT	ATACCTTGAGCATCAGTTGG
KlSOD2	CAATTCGGCTCTCTAGACAA	AGTAAGCATGTTCCCAAGAG

Table S1: Primer sequences used in the yeast real-time PCR experiments.

6. EGTA challenge assay

Cells grown until late exponential phase were challenged with 20mM Ethylene glycol-bis (2- aminoethylether) -N,N,N2,N2-tetraacetic acid (EGTA) for 6h. Afterwards, aliquots of the cells were withdrawn, diluted and plated on agar plates. EGTA sensitivity was assessed by CFU counting method and results were expressed as percentage of cell

survival with respect to untreated samples. The data are the mean of three different experiments.

7. ADH activity analysis

Yeast cells were grown to the early stationary phase in 10 ml of SD medium supplied with the appropriate auxotrophies. Cells were broken with glass beads in Eppendorf tubes, centrifuged and the supernatants were analyzed by electrophoresis on non-denaturing 5% acrylamide gel.
ADH isozymes were revealed by incubating the gels for 1 hr at 23° C in the dark with a mixture containing 0.5 M ethanol; 7 mg/ml NAD; 25 μg/ml phenazine methosulfate (Sigma); 1 mg/ml nitroblue tetrazolium chloride in 60 mM pyrophosphate buffer, pH 8.8.
The activity of the Glucose-6-Phosphate Dehydrogenase (G6PDH), used as positive control, was visualized by incubating the gel for 10 to 15 min in 5 ml of the following solution: 10 μl of 1 M $MgCl_2$, 50 μl of NADP (100 mg/ml in 100 mM Tris-HCl, pH 8.0), 15 μl of phenazine methosulfate (40 mg/ml in H_2O) (Sigma), 30 μl of nitroblue tetrazolium salt (50 mg/ml in H_2O) (Sigma), 25 μl of glucose-6-phosphate (100 mg/ml in H_2O), and H_2O to a final volume of 5 ml.

8. Quantitative Real-time PCR from keratinocytes

Total RNA from primary cells was extracted using Trizol Reagent (Thermo Fisher Scientific, MA, USA) according to manufacturer's instructions. Reverse transcription (RT) of total RNA was performed using High capacity cDNA Reverse transcription kit as previously described (Cialfi et al., 2013) (Thermo Fisher Scientific, MA USA) and according to manufacturer's instructions. Quantitative Real time PCR (qRT-PCR) for GST-M1, GST-T1 and NRF2 expression analysis were carried out using specific primers indicated in Table S2 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). GAPDH mRNA was used as endogenous control to normalize sample data. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA amount of lesion compared to not lesion sample represented as unitary value.

GENE	SEQUENCE 5'-3'	PCR PRODUCT
GSTM1 qPCR Fw	AGAGGAGAAGATTCGTGTGG	182 bp
GSTM1 qPCR Rev	TGTTTCCTGCAAACCATGGC	
GST-T1 qPCR Fw	AAGGTCCCTGACTACTGGTA	151 bp
GST-T1 qPCR Rev	ATACTGGCTCACCCAGGAAA	
GAPDH qPCR Fw	*TGCACCACCAACTGCTTAG*	176 bp
GAPDH qPCR Rev	*GAGGCAGGGATGATGTTC*	
Taqman gene expression assay	***Assay reference number***	
NFE2L2 (NRF2)	Hs00975961_g1	
GAPDH	Hs02758991_g1	

Table S2: Primes used for the qRT-PCR

9. Screening of an FDA-approved compound library

A library of 131 FDA-approved drugs was purchased from Selleck Chemicals (Houston, TX, USA). Compounds were stored as 10 mM stock solutions in dimethyl sulfoxide (DMSO) at -20°C until use. Compound stocks were diluted to the utilized concentration in the growth YPD medium. After 24h of growth at 28°C the cells were harvested and prepared for the CFW or DASPMI staining.
Alternatively, for the sensitivity tests, yeast strains were grown for 24h in the YPD medium; than five-fold serial dilution from concentrated culture were spotted onto synthetic YPD agar plates supplemented or not with 20mM EGTA or 4mM H_2O_2 or 60 μM menadione . The plates were incubated at 30°C for 3 days.

10. Worm strains and media

The worm strains used in this work are the standard wild type N2 (Bristol) and LD1 [*ldIs7*{P_{skn-1}::skn-1B/C::GFP+pRF4(*rol-6*(su1006))}], purchased from the *Caenorhabditis* Genetics Center (CGC).

Worms were grown on Nematode Growth Media (NGM) plates at 20°C. NGM contains: 3 g/L NaCl, 17 g/L agar, 2.5 g/L peptone, 1 ml/L cholesterol (5 mg/ml in 95% EtOH), 1 ml/L 1 M $CaCl_2$, 1 ml/L 1 M $MgSO_4$, 25 ml/L 1 M KH_2O_4 buffer pH 6. The plates were dried and *E. coli* OP50 strain was spread to serve as worm food. Seeded plates were incubated at 37°C overnight and used after a time of about 30 min at room temperature.

11. Construction of the RNAi strains

Based on the nucleotide sequence of ZK256.1c (Chromosome I: 13004437-13023625; Worm Base), forward (CEf 5'-CCGCCAATTCACAGTTTGGA-3') and reverse (Cer 5'-CATTCTGCCCATGTGCATCA-3') primers were designed to amplify a *pmr-1* fragment from genomic DNA by PCR, using the BIOTAQ DNA polimerase (Bioline). The PCR product was ligated into the pGEM-T-Easy vector (Promega) and controlled by EcoRI (Roche) digestion. After sequencing (MWG Biotech, Ebersberg, Germany) the *pmr-1* ORF was cloned in XbaI/PstI -digested L4440 double promoter vector (Timmons *et al.*, 2003), to generate a *pmr-1* double-strand RNA (dsRNA) expression plasmid (L4440-*pmr-1*). The resulting plasmid was used to transform the HT115(DE3) RNase Ill-deficient *E. coli* strain, which was shown to be beneficial for RNAi by feeding (Timmons and Fire, 1998). Moreover the HT115 *E. coli* strain transformed with the "empty" L4440 vector was used as control in the RNAi expreriments.

The HT115 genotype is the following: (*F*$^-$, *mcrA, mcrB, IN(rrnD-rrnE)1, lambda*$^-$, *rnc14::Tn10(DE3 lysogen:lacUV5 promoter-T7 polymerase*)). The RNase III gene is disrupted by a Tn10 transposon carrying a tetracycline-resistance marker.

12. Preparation of RNAi plates

Single colonies of HT115 bacteria transformed with the plasmid were picked and grown overnight at 37°C, supplemented with 50 μg/ml Ampicillin and 12,5 μg/ml Tetracycline. Afterward, 1ml of overnight cultures was inoculated in 100 ml of *Luria-Bertani* (LB) broth supplemented with 50 μg/ml Ampicillin and incubated at 37°C to OD_{600}=0,4; then 0,4 mM IPTG was added to the cultures for 3h.
When the induction time finished, the culture were pulled down by centrifugation, resuspended in 2 ml of distillated water, spread on RNAi NGM plates and then left at 37°C overnight.
The RNAi NGM plates contain 3 g/L NaCl, 17 g/L agar, 2.5 g/L peptone, 1 ml/L cholesterol (5 mg/ml in 95% EtOH), 1 ml/L 1 M $CaCl_2$, 1 ml/L 1 M $MgSO_4$, 25 ml/L 1 M KH_2O_4 buffer pH 6, 0,4 mM IPTG, 50 μg/ml Ampicillin, 12,5 μg/ml Tetracycline.

13. Life span analysis

The life span assays are performed at 20°C with synchronized L4 wild-type worms fed on bacteria expressing *pmr-1* RNAi or the empty vector *EV* (RNAi) for 48h. After this time, the animals were shifted on RNAi plates containing 20 mM EGTA, and thus the life span started (To). The worms were transferred to fresh plates and monitored daily. The nematodes were scored as dead when they no longer responded to gentle prodding with a platinum wire. Animals that crawled off the plates were not included in the analysis.

14. Worm imaging

Synchronized LD1(*skn-1*::GFP) worms at the stage of one day-adult were fed with *EV*(RNAi) or with *pmr-1*(RNAi) for 48h and 72h. Besides, to remove the bacteria on the animal body the worms were

washed twice with M9 buffer (3g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5g/L NaCl, 1 ml 1M 1 M $MgSO_4$ dissolved in distillated water). Microscopy images were taken mounting the washed animals on a 3% agarose plus 20 mM sodium azide pad on a microscopic glass slide and observed with a Zeiss Axiovert 25 microscope.

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