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PhD Thesis

Tesi di Dottorato

"Ubiquitin-proteasome dependent regulation of p73 protein stability"

"Regolazione ubiquitina-proteasoma dipendente della stabilità proteica di p73"

PhD Student:

Flavia Scialpi

Thesis advisors:

Prof.GM Lauro (University of Rome "Roma Tre")

Dr. Francesca Bernassola (University of Rome "Tor Vergata")

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1. Introduction

1.1 Overview of the ubiquitylation process

Post-translational protein modifications are used to generate and relay signals in almost every cellular pathway. Maybe the best understood modification is protein phosphorylation, which has numerous consequences, although other post-translation protein modification (i.e. acetylation, ubiquitylation and methylation) are thought to be more limited, having specific roles. In this view, the conjugation of a target protein with the small globular protein called ubiquitin, also named "ubiquitylation", is best known as a signal for controlled protein degradation; conversely, recent evidences suggest that ubiquitin generates a signal which can be as far-reaching as those produced by phosphorylation, also because of the different types of ubiquitin modification that can be observed in living cells, that have potential diverse signal outcomes. Thus, ubiquitylation is chemically more complex than post-translational modification such as phosphorylation.

The ubiquitin, discovered in 1975 as a 8.5-kDa protein of unknown function, owns this name because of it is ubiquitously expressed in all eukaryotic cell types; moreover, it is highly conserved, but absent from bacteria and archea, with changes in only three amino-acid over the sequence from yeast to human.

Ubiquitylation is a multistep process which concerns upon all aspects of eukaryotic biology: it is now widely known that different types of ubiquitin linkage can trigger diverse biological outcomes (see below). Whenever ubiquitylation process is defective or deregulated, a large range of diseases could arise, from developmental abnormalities to neurodegenerative diseases, from autoimmunity to cancer.

The ubiquitylation process was initially characterised in cellular extracts as an ATP-dependent proteolytic system in which a heat-stable polypeptide named APF-1 (ATP-dependent proteolysis factor 1) was found to become covalently attached to a protein substrate in an ATP- and Mg^{2+}-dependent process. It was found that multiple APF-1 molecules were linked to a single substrate molecule

by an isopeptide linkage, and that successively these conjugates were been rapidly degraded, observing a release of free APF-1 (Hershko et al., 1979; Ciechanover et al., 1980; Wilkinson et al., 1980). Soon after APF-1-protein conjugation was characterised that protein was identified as ubiquitin, and then it was been enlightened that the carboxyl group of the C-terminal glycine residue of ubiquitin (Gly76) is involved in conjugation to substrate.

Prokaryotes do not have a molecule functionally related or analogous to ubiquitin. This means that in prokaryotes there isn't a protein that acts as a signal through covalent binding to a target substrate, even though they possess proteins that display an ubiquitin fold. A good example is the *E. coli* ThiS protein, a sulfur carrier which shares only 14% sequence identity with ubiquitin, but which possesses the ubiquitin fold, suggesting that the eukaryotic ubiquitin and the prokaryotic ThiS could be evolved from a common ancestor (Wang C et al., 2001). During evolution a number of eukaryotic covalently attachable proteins that share ubiquitin fold has been generated (also named ubiquin-like proteins, UBLs). In addition to folding, UBLs share the capacity to be bound through an isopeptide bond, formed between the modifier's terminal glycine and an amino group of the target protein. Usually the amino group is contributed by a lysine residue, but N-terminal ubiquitiylation is also known (Bloom et al., 2003). Moreover, UBLs also display a strict amino acid sequence homology: for example, Sumo-1 is 20% identical to human ubiquitin and 52% identical to yeast Sumo (Smt3). Strong interspecies sequence conservation indicates that the biological functions of those proteins is conserved (**Fig. 1**). The best known UBLs, Sumo and Nedd8, are universally distributed in eukaryotes, whereas other ones are found only in mammals.

Even if much remain unknown, studies carried out in last several years have shown a remarkable progress in elucidating the UBLs functions as for sumoylation, that regulates nucleocytoplasmatic transport and cell cycle progression by modulating localization and/or activity of its subsytrates. On the contrary, it is well known that there is a common biochemical mechanism by which these functions are carried out: the modifiers are conjugated to an amino group of target proteins, leading to several specific downstream events. Sometimes, as for ubiquitin and Sumo, the conjugation of the second one can preclude ubiquitylation and subsequent consequences (i.e. Hoege et al., 2002). The best

example of cross-talk between ubiquitin family members is provided by Nedd8 (yeast Rub1), that modifies a class of enzymes involved in ubiquitylation stimulating these enzymes to become more active in linking ubiquitin to substrates (see below, rewived in Hochstrasser, 2000).

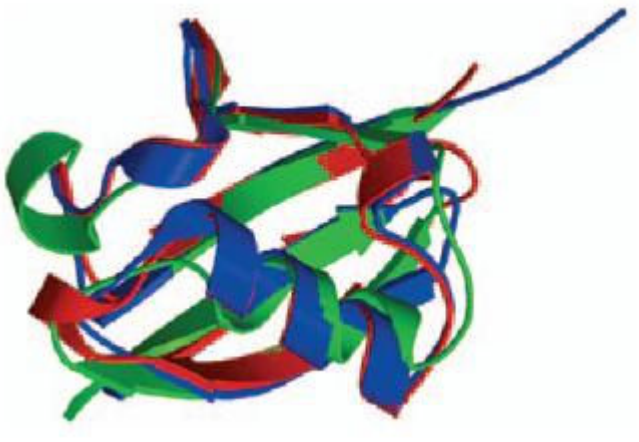


Fig.1 - The ubiquitin superfold and UBLs. Ubiquitin and its relatives are related primarily by the ubiquitin superfold, which is a β-grasp fold. The figure shows an overlay of ubiquitin (blue), SUMO-1 (green) and NEDD8 (red) *(Welchman et al., 2005, modified)*

The best-studied role of covalent ubiquitylation (also called ubiquitination) concerns degradation of proteins whose levels are regulated either constitutively or in response to changes in cellular environment. The crucial role of this conjugation can be observed in myriad processes as cell cycle progression, regulation of cell proliferation, cellular differentiation, organelle biogenesis, quality control in the endoplasmic reticulum, protein transport, apoptosis, antigen processing, inflammation, DNA repair and stress responses, explaining why ubiquitin could be involved in so much diseases. The canonical ubiquitin signal, a poly-ubiquitin chain brought forth linkages between the terminal Gly of an ubiquitin molecule and the

Lys48 of the following one, is recognized by the multisubunit protease termed 26S proteasome as a signal for degradation of tagged proteins. The most fascinating aspect of the process is that this tagging consists in a fine and rapid method for controlling protein abundance in those processes that require a selective and rapid destruction of proteins, for example when a switch-like signal is needed, as for transition between stages of the cell cycle. But by the side of proteasome-dependent degradation, other roles for ubiquitylation are emerging, outlining ubiquitin as the same signal for fundamentally different proteolytic structures and other cellular functions not directly involving protein degradation (i.e. destruction through vacuoles and/or lysosome pathway, DNA repair and regulation of translation).

It isn't well understood how the same signal can settle so different destiny, as protein destruction versus enhanced translation. Specificity is generated largely by the enzymes that recognize substrate and mediate ubiquitylation, but it's also important, to determine the fate of an ubiquitylated protein, what type of ubiquitin conjugate is formed (see below) **(Fig. 3)**. Basically, it's now evident that a single ubiquitin tag doesn't target a protein for degradation, whereas a chain of four or more residues does (Thrower et al., 2000). In addition, there are subtly different ways of building a multi-ubiquitin chain, by using different internal residues of ubiquitin itself (see below). The intracellular location of ubiquitylated proteins also helps to define their fates, meaning that ubiquitylation in the nucleus might not have the same consequences as that in the cytosol, as it can be logically supposed.

Ubiquitylation is a dynamic and reversible process: within cells de-ubiquitylating enzymes are responsible for removing ubiquitin from proteins and disassembling multi-ubiquitin chains. These enzymes are also important in the ubiquitin maturation process, because this small protein is encoded on multiple genes and translated as fusion proteins either with other ubiquitin molecules or as the N-terminal component of two small ribosomal subunits, then processed by ubiquitin carboxy-terminal hydrolases in order to have mature ubiquitin (Finley et al., 1989) (**Fig. 2**).

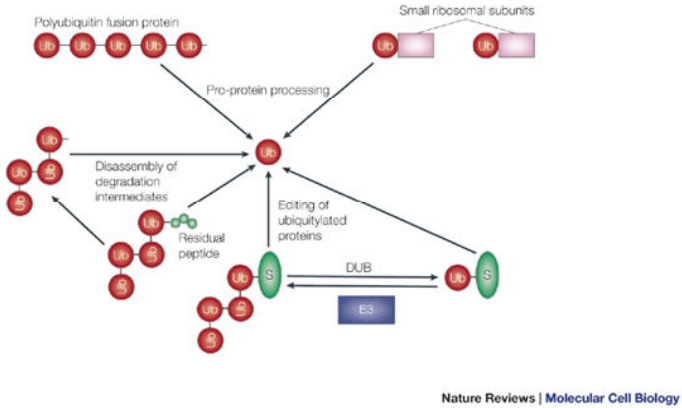


Fig. 2 – Ubiquitin maturation process and recycling. *(from Weissman, 2001)*

Ubiquitin has seven lysine residues; in addition to Lys48, at least four of these (Lys6, Lys11, Lys29 and Lys63) can function as a linkage for poly-ubiquitin chains (Johnson et al., 1995; Schneel & Hicke, 2003) (**Fig. 3**). Not all modifications obtained with the conjugation of ubiquitin moiety mean "proteolysis": they can be non-proteolytic and reversible events such as changes in protein activity, protein-protein interactions and subcellular localization; furthermore, ubiquitylation is not always personified by chains: the conjugation of a single ubiquitin to a target protein is known as monoubiquitylation, whose aim is to regulate diverse processes including histone fuction, transcription, endocytosis and membrane trafficking (Hicke, 2001; Katzmann et al., 2002) **(Fig. 3)**. In particular, non-proteolytic Lys6 and Lys11 polyubiquitin linkages have been correlated with pathogenesis of neurodegenerative disorders (Bennett et al., 2007; Cripps et al., 2006), while single or multiple monoubiquitylation of cell surface receptors triggers to their internalization and trafficking to the endosomal degradation pathway (reviewed in Di Fiore et al., 2003).

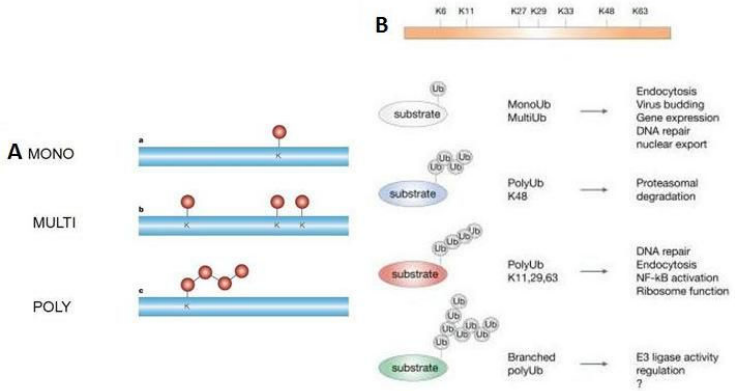


Fig. 3– Overview of ubiquitin-conjugation fashions. A. Target proteins can be differently modified by binding of one or more molecules of ubiquitin, in different fashions **B.** Ubiquitin amino acid sequence contains seven lysine employed in different kinds of chain, with various but sometimes similar outcomes *(from Woelk et al., 2007; Hicke, 2001, modified)*

1.1.1 Biochemistry of ubiquitylation: E1, E2 and E3 enzymes

As mentioned above, the ubiquitylation of target proteins is a multistep process. The steps of ubiquitylation are constituted by biochemical reactions catalyzed by three classes of enzymes, named E1, E2 and E3 (**Fig. 4 and 5**). Firstly, an ubiquitin-activating enzyme (also known as E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP-dependent manner; then, a ubiquitin-conjugating enzyme named E2 (as well as UBC) gets ubiquitin from E1 by a trans-esterification reaction, involving itself and the carboxy terminus of ubiquitin, again. Finally, the last but not least protagonist of the process is an ubiquitin protein ligase enzyme (E3), which catalyzes the transfer of ubiquitin from E2 to target protein, involving the ε-amino group of a lysine residue on the target (Hershko and Ciechanover, 1994) (**Fig. 4**).

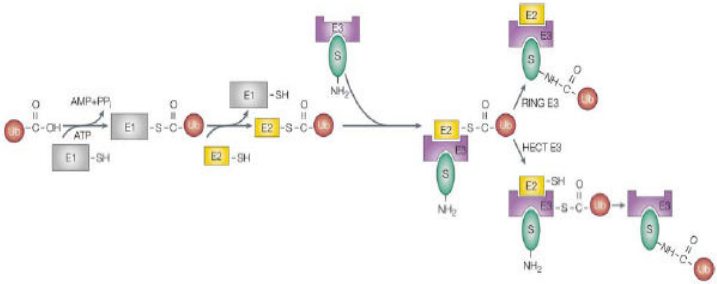


Fig. 4 – Ubiquitylation is a multistep process. *(from Fang and Weissman, 2004)*

There are more E2s than E1s, and more E3s than E2 so, at each step, the number of proteins that can potentially be involved increases, as the specificity of binding to the next component does, althought it's the E3, either alone or in combination with E2, that determines the fine specificity of substrate recognition. **(Fig. 5)**.

In general, each UBLs has a single dedicated E1. this is true even for ubiquitin (except in plants). E1 is found in the nucleus and cytosol and associated with the cytoskeleton (Trausch et al., 1993; Grenfell et al., 1994). E1 is also phosphorylated in mammalian cells by the cyclin-dependent kinase Cdc2 (Nagai et al., 1995), which presumably reflects the crucial role of the ubiquitin conjugation pathway in cell cycle control.

It's generally believed that a single essential E1 enzyme is involved in ubiquitylation; several E1 genes from different species have been characterized and code for proteins of 115-125kD. In mammals two isoform of E1 enzyme have been found (referred as E1a and E1b), arising from the utilization of two translation initiation sites (Handley-Gearhart et al., 1994). Wheat, and possibly other organisms, contain genes for more that one E1 (Hatfield & Vierstra, 1993). The role of these different E1s is not understood.

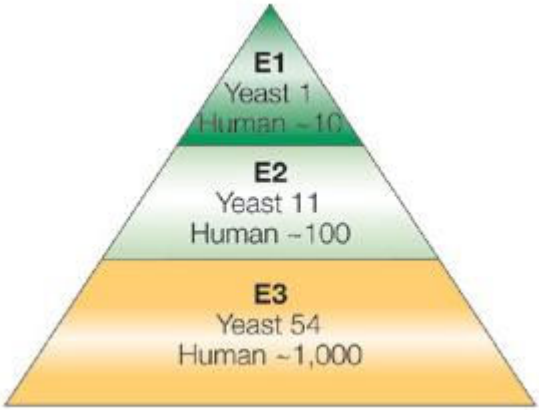


Fig. 5 – Complexity of ubiquitin process enzymes. Approximative numbers of known and predicted genes coding for ubiquitylation process enzymes are indicated *(from Hicke et al., 2005)*

While there is only one E1 enzyme in yeast, *S. cerevisiae* is currently known to contain 13 genes coding for ubiquitin-conjugating enzymes (E2), suggesting that at least some of the specificity of ubiquitinylation is dependent on E2s. Two of these 13 enzymes are E2s for SUMO (Ubc9) and Nedd8 (Ubc12) (Hochstrasser, 2000). Mammalian genomes include over 30 E2 enzymes (von Arnim, 2001). With few exceptions, E2s range from 14 to 36 KDa. All these enzymes contain a cysteine in a conserved domain of about 16kD called "UBC domain" because of the UBC motif which can be found within. The UBC domain is modelled as a shallow cleft on protein surface. The conserved cysteine is required for E2 activity, and accepts ubiquitin from E1 to form a thiol-ester. Some E2s have substantial amino- or carboxyl-terminal extensions which may regulate E3 association, intrinsic E2 activity and substrate recognition, whereas some other have insertion in the UBC (Pickart, 2001). Similarly, the amino acid composition in regions of contact between E2s and E3s may affect productive E2-E3 interactions. Based on the number of potential/effective E3s protein sequences, E2s would be predicted to function with multiple E3s. At least *in vitro*, many E3 also have the capacity to function with

multiple E2s; however, the number of pairings are clearly restrincted. Noncovalent E2/ubiquitin binding affinities are usually low, highlighting the important role that E1 plays in bringing the E2 together with ubiquitin. The C-terminus of ubiquitin (residues 71 to 76) adopts a partially extended conformation that wraps around part of E2 surface, occupying the cleft formed by specific E2 residues, but does not overlap the site where E3 enzymes are expected to bind (Hamilton et al., 2001).

The most extraordinary feature of ubiquitin conjugation pathway is the wide breadth of substrates and related ubiquitin biological functions, strictly linked to the large number of existing E3 enzymes. In fact, target specific recruitment is exerted by this class of enzymes. A comparison to phosphorylation is effective: just as in a cell there are numerous kinases, each with a limited suite of substrates, so as many E3 can be found, each alike with a finite set of target proteins. In both cases, the pairing enzyme/substrate explains the elegant specificity in regulating substrate modification: the E3 binds the target protein, effectively selecting the target. This implies E3 recognize a motif in the substrate protein that targets it for ubiquitinylation. It has been suggested to call such motifs "degrons", meaning any motif that targets proteins for degradation (by the ubiquitin-dependent or and other systems).

It could be wondering why E3 do not acquire ubiquitin directly from E1. Two answers have been provided, both related to regulatory potential. The first one can be guessed viewing the multi-step nature of the process as a skill to finely regulate it. In fact, if a given E3 functions mainly with one E2, the E2 step provides an additional point of regulation, for example changing E2 activity or concentration within the cell. Moreover, the existence of many E2 may further diversify the specificity of ubiquitylation, relating a given E2 to the modulation of target protein selection or to the structure of ubiquitin modification (i.e. through Lys48 rather then Lys63 linkages). Substrates whose ubiquitylation can involve multiple E2s can exemplify this second strategy.

Until recently, the donor of ubiquitin to target proteins was thought to be an ubiquitin charged E2, and E3s were not thought to be involved in the transfer of ubiquitin to the substrate protein. Originally, E3s were proposed as bridging factors acting between E2s and substrates (Hershko et al., 1983). This has been turned into

the present view by accurately defining structure and functions of E3s ligases on a bioinformatics basis.

The best known E3s belong to two protein families:

1. **H**omologous to **E**6AP **C**arboxy **T**erminus (HECT ligases), which do form thiol esters with ubiquitin.
2. **R**eally **I**nteresting **N**ew **G**ene (RING ligases), which do not form thiol esters with ubiquitin.

HECT ligases (HECTs), although significant in size, are much smaller than the RING family members (RINGs). The total number of E3s in higher organisms ranges around several hundreds; the capability to generate so many E3s from just three protein domain lays by the modular construction of E3 enzymes. The HECTs and the RINGs share a common property of E2 binding, grafted onto different domain(s).

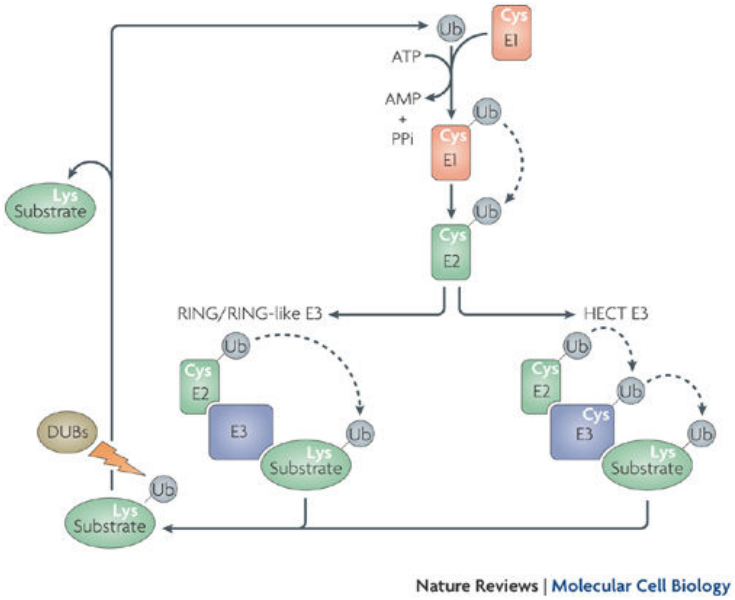


Fig. 6 – The ubiquitin-conjugation machinery *(from Ravid & Hochstrasser, 2008)*

The main difference among ligases belonged to HECT and RING families is due to HECTs capacity to catalyze activated ubiquitin transfer from E2 enzyme to the target protein, whereas RINGs cannot: they steer E2 to specific target indeed **(Fig. 6)**. Basically, HECT ubiquitin ligases function themselves as ubiquitin carriers. Others specific domain structurally related to RING domain are recently be identified: another Zn-binding domain called the PHD finger (Coscoy & Ganem, 2003), and the U-box domain (Ohi et al., 2003).

1.1.4 HECT E3s

As described by the name, the main feature of HECT E3 ligases subfamily is represented by the HECT domain, a large C-terminal module of approximately 350 amino acids. This domain has been first characterized in the E6-associated protein (E6-AP), and it is responsible for the association with E2 enzyme as well as the catalytic activity (Huibregtse et al., 1995). HECT E3s have been found from yeasts to humans and range in size from approximately 80 to more than 500 KDa.

The HECT domain is composed by two lobes, a larger one containing the E2 binding site and a smaller one including the Cys residue that forms thioester complexes with ubiquitin and so it is responsible for its catalytic activity. These two regions are connected by a hinge which is the key feature of the domain to appropriately set E2 and E3 catalytic residues during the ubiquitin transfer (Verdecia et al., 2003).

HECT ligases own protein-protein interaction domains located within N-terminus that dictate for substrate recruitment specificity and that have been taken on account to classificate them into three subfamilies (Scheffner & Staub, 2007, **Fig 7**):

1. HERC E3, containing mitotic regulator RCC1-like domains (RDLs)
2. C2-WW-HECT E3s, containing Trp-Trp domains (WW domains), also termed Nedd4-like HECT E3s
3. SI(ngle)-HECT E3s, lacking either RCC1 or WW domains.

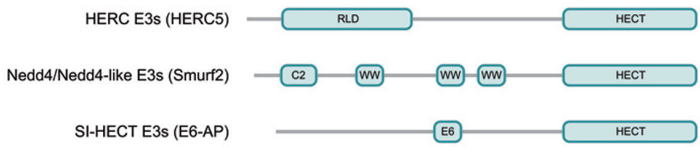


Fig. 7 –HECT E3s subfamilies. All members of the HECT E3 family are characterized by the C-terminal HECT domain. The HERC family comprises six members characterized by the presence of one or several RLD domains. The Nedd4/Nedd4-like family has nine members characterized by an N-terminal C2 domain and several WW domains. On the contrary, the members of SI-HECT E3s subfamily are characterized by the notion that they contain neither RLDs nor WW domains. *(from Scheffner & Staub, 2007)*

Due to the identification of several tumor suppressors as substrates of HECT E3 ligase (summarized in **Fig. 8**), as well to the discovery of genetic aberrations of some of the family members in human cancer, it's logical to suppose that HECT-type E3s can have a oncogenic potential, which actually has been extensively investigated.

The C2-WW-HECT subfamily represents the best characterized subgroup of HECT ligases, highly conserved through evolution from yeast to mammals. In humans, this subfamily includes nine members with respective orthologs in mice. These proteins share a common structure which can be summarized in a N-terminal protein kinase C (PKC)-related C2 domain, two to four WW domains, and a C-terminal HECT domain (Schwarz et al., 1998; **Fig. 9**). The C2 domain takes its name because of its capability to bind Ca^{2+}; it is also involved in localizing the HECT E3s to intracellular membranes through the binding to phospholipids (Dunn et al., 2004). The other pivotal feature of this subfamily is represented by WW domains, that mediate ligase-substrate associations through interaction with a proline rich motif PPXY (PY) consensus sequence, contained in the substrates. Another consensus sequence, represented by phosphoSer/phosphoThr followed by a proline residue, can be recognized by WW domains.

Also interactions with adaptor and regulatory proteins have been described, though they also own PY motifs.

E3	Substrate(s)	Outcome of Substrate Ubiquitylation	Adaptors/Regulators	Biological Function
E6-AP	p53	proteasomal degradation	E6	apoptosis
Huwe1	p53	proteasomal degradation	ARF	apoptosis, growth arrest
EDD	TopBP1	proteasomal degradation	unknown	DNA damage
Nedd4-1	PTEN, Hgs, Eps15	proteasomal degradation, cytoplasmic/nuclear shuffling	unknown	apoptosis, genome integrity, endocytosis
Nedd4-2	Smad2, Smad4, TβR-I/II	proteasomal degradation	Smad6, Smad7	apoptosis, growth arrest
Itch	p73, p63, Notch1, c-Jun	proteasomal degradation	Numb	apoptosis, differentiation
WWP1	p53, Notch1, KLF2, KLF5, Smad2, Smad4, TβR-I/II	proteasomal degradation, nuclear export	Smad2, Smad6, Smad7	apoptosis, growth arrest
Smurf1	Smad1, Smad4, Smad5, TβR-I/II, BMP-RI/II	proteasomal/lysosomal degradation	Smad6, Smad7	apoptosis, growth arrest
Smurf2	Smad1, Smad2, Smad4, Smad5, TβR-I/II	proteasomal/lysosomal degradation	Smad2, Smad7	apoptosis, growth arrest

TopBP1, topoisomerase IIβ binding protein; BMP-R, bone morphogenetic protein receptor.

Fig. 8 – The HECT family of E3s and their substrates. *(from Bernassola et al., 2008, modified)*

The C2-WW-HECT E3s are involved in several pathways including endocytosis, degradation of membrane proteins and control of cell growth (Scheffner & Staub, 2007). HECT E3 ligases usually regulate endocytosis and trafficking of plasma membrane proteins (via monoubiquitylation) and stability of transmembrane and cytosolic substrates (via polyubiquitylation). They are subjected to fine regulation exerted through posttranslational modification and/or interaction with adaptors proteins (Kee & Huibregtse, 2007). In the context of this thesis work, I will focus on Itch, a member of this C2-WW-HECT E3 subfamily (see below).

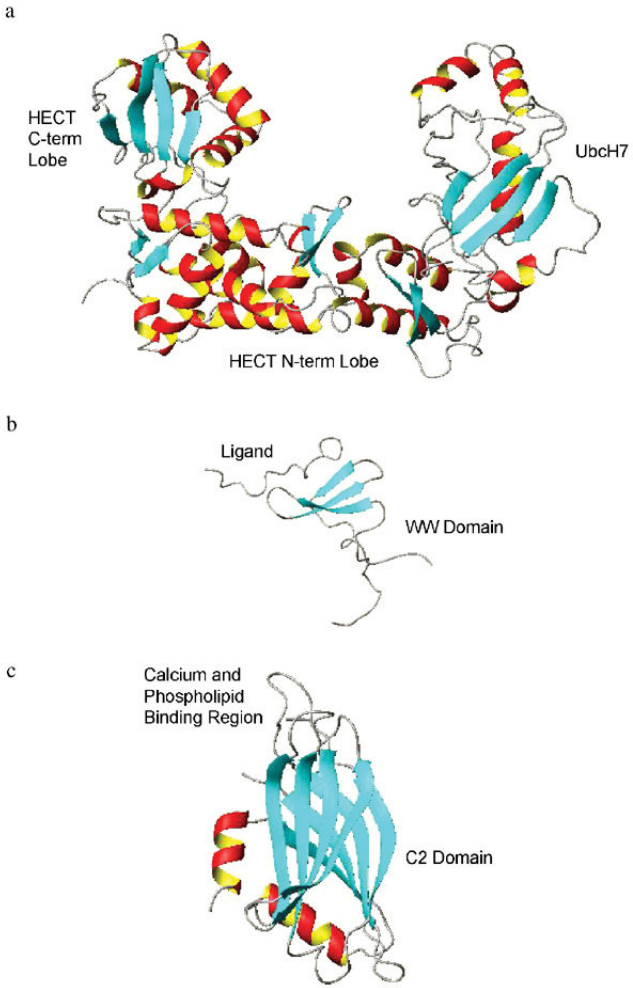


Fig. 9 – Molecular structure of C2-WW-HECT E3 ubiquitin ligases domains. A. the HECT domain bound to E2 ubiquitin ligase UbcH7 **B.** a single WW domain **C.** the C2 domain *(from Ingham et al., 2004)*

Itch

The E3 ubiquitin ligase Itch (also referred to as AIP4, Atrophin-1 Interactin Protein 4), was originally identified in 18H *agouti* mice through genetic studies carried out to investigate mutations in the *agouti* locus. These mutations result in alterations of coat color, demonstrated to be caused by a chromosomal inversion induced by radiation and associated with a deletion of proximal and distal inversion breaks (of 18 and 20 base pairs, respectively). This inversion causes the loss of expression of two genes, *agouti* and *Itch*. This latest gene codifies for a protein of 854 amino acids, which includes a HECT, four WWs domains and a N-terminal C2 domain, hence it has been classified as a member of C2-WW-HECT subfamily of HECTs.

The non-agouti-lethal 18H mice display inflammatory and immune defects associated with a persistent scratching of the skin. Specifically, Itch deficiency results in a systemic autoimmune-like disease which shows a late onset and which is progressively lethal, characterized by lymphoproliferation in spleen, lymph nodes and thymus medulla, and by chromic pulmonary interstitial inflammation (Perry et al., 1998).

Itch regulates the protein stability of both transmembrane receptors (through monoubiquitylation or multiubiquitylation) and intracellular substrates (through polyubiquitylation), driving them to lysosomal and proteasomal degradation, respectively. Proteolysis-independent ubiquitylation events have also been ascribed to Itch E3 ligase activity. Although a small fraction of Itch is localized perinuclearly (overlapping the trans-Golgi network), the protein is predominantly associated with early and late endosomal compartments and lysosomes, due to its C2 domain (Angers et al., 2004; Marchese et al., 2003).

Although initially thought to be constitutively active, the number of evidences ascribed to functional regulation of HECTs has become increasing (i.e. Debonneville et al., 2001; Ogunjimi et al., 2005; Chen et al., 2005), even if generally phosphorylation does not appear to alter the catalytic activity of the HECT domain, but rather to reduce the ability of E3s to associate with their substrates. Itch E3 activity is regulated by phosphorylation, as positively, through MEKK1/JNK1 pathway, as well negatively, through Fyn kinase-mediated tyrosine phosphorylation (Yang et al., 2006; Gao et al.,

2004). After T cell receptor engagement, it has been shown that Itch undergoes JNK1-mediated phosphorylation that enhances its enzymatic activity. This phosphorylation concerns three residues within Itch (S199, S232, and T222), located within a Pro-rich region. JNK1-mediated phosphorylation disrupts an inhibitory interaction between the WW and HECT domains of Itch, inducing a conformational change that strongly enhances the catalytic activity of Itch (**Fig. 10**).

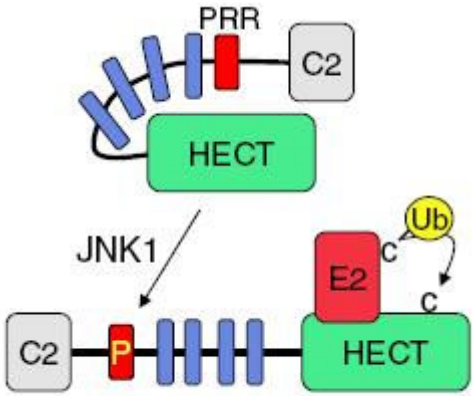


Fig. 10 – Phosphorylation-dependent activation of Itch. Itch adopts a "closed" inactive conformation due to intramolecular interactions between the WW-PRR region and the HECT domain. In response to signaling events, JNK1 is recruited and then phosphorylates multiple sites within the PRR region. This relieves the inhibitory interactions, leading to activation of the E3. *(from Kee & Huibregtse, 2007, modified)*

Itch catalytic activity can be regulated also by interaction with adaptor proteins, which contribute to modulate their substrate recruiting capacity, subcellular localization, and enzymatic activity. Itch-interacting proteins can enable further specificity to the ubiquitylation reaction, as explained by the following examples.

N4BP1 is a novel Itch negative regulator, acting as a competitor of its substrate recruitment ability. Although N4BP1 does not contain canonical WW domain consensus sites, it is able to bind to these domains determining the displacement of the substrates from the ligase, preventing Itch-mediated ubiquitylation of targets (Oberst et al., 2007).

Nedd4 family interacting protein-1 (Ndfip1) is a membrane associated protein which possesses two N-terminal PY motifs, mediating direct interaction with Nedd4 as well as Itch. In mammals, such interaction is responsible for promoting Itch-mediated degradation of target substrates that could bind Ndfip1 but not directly Itch. In particular, after T-cell stimulation, Itch is relocalized and Itch-mediated ubiquitylation and degradation enhanced. As a result, T cells produce TH2 cytokines and promote TH2-mediated inflammatory disease (Oliver et al., 2006).

Another Itch protein interactor which is responsible for modulating Itch activity is the mammalian homolog of Drosophila Numb. Numb protein, whose phosphotyrosine-binding (PTB) domain recruits the intracellular domain portion of Notch, cooperatively enhances Itch-catalyzed ubiquitylation of the membrane bound receptor, and specifically promotes the ICD degradation following receptor activation (see below) (McGill & Mc Glade, 2003). This effect is achieved through direct binding of Numb to Itch WW1/2 domains. Also the Itch-mediated ubiquitylation and degradation of transcription factor Gli is regulated by. Numb. Gli mediates the effect of Hedgehog signaling in neural stem cell maintenance and self-renewal. Since accumulation of Gli in the nucleus is the major mechanism regulating its transcriptional activation, Numb-dependent stabilization of Itch-Gli complex stimulates Gli ubiquitin-dependent proteasomal proteolysis. Thus, Numb acts as an antagonistic regulator of Hedgehog signaling during cerebellar development (Di Marcotullio et al., 2006).

Different Itch substrates have been identified so far, many of which are important factors regulating immune response, TGF-β signalling, receptor trafficking and signalling, epidermal keratinocyte differentiation and cell death (**Fig. 11**). Interestingly, Itch itself is a substrate of its E3 ligase activity, given that autoubiquitylation activity has been reported (Gao et al., 2004; Mouchantaf et al., 2006; this thesis). More recently, it has been

identified the ubiquitin-protease FAM/USP9X as a binding partner of Itch. Transient overexpression of FAM/USP9X resulted in the deubiquitylation of Itch and in an increase of Itch levels, suggesting that Itch could be degraded by the ubiquitin-proteasome pathway (Mouchantaf et al., 2006).

Substrate	Function	Biological outcome	Regulators/ adaptors
c-Jun	Transcription factor	Regulation of T_H2 cell differentiation/anergy	JNK, N4BP1
Jun-B	Transcription factor	Regulation of T_H2 cell differentiation/anergy	JNK, Fyn, Ndfip1
PLC-γ1	Phospholipase	Regulation of T-cell anergy	Unknown
PKC-θ	Kinase	Regulation of T-cell anergy	Unknown
Notch	Transcription factor	Regulation of autoimmunity	Numb
Gli	Transcription factor	Repression of Hedgehog signaling	Numb
Deltex	Regulator of Notch signals	Regulation of autoimmunity	Unknown
Smad2	Receptor-activated Smad	Activation of TGFβR signaling	Unknown
TIEG1	Transcription factor	Activation of TGFβR signaling	Unknown
P73	Transcription factor	Regulation of apoptosis, neural development, cancer	N4BP1
P63	Transcription factor	Regulation of apoptosis, epithelial development, cancer	N4BP1
c-Flip	Apoptosis inhibitory protein	Regulation of apoptosis	JNK
ErbB4	Growth factor receptor	Epithelial kinase receptor, cancer	Unknown
Endophilin A1	Protein of the endocytic machinery	Regulation of EGFR endocytosis	Unknown
CXCR4	Chemokine receptor	Agonist-dependent sorting of G protein-coupled receptors	Unknown
Hsr	Protein of the endocytic machinery	Regulation of cargo sorting	Unknown
TRPV4, TRPC4	Cation channels	Regulation of channel recycling and abundance at the cell surface	Unknown
Itch	E3 ubiquitin ligase	Control of protein stability, potential regulation of E3 catalytic activity	USP9X/FAM

Fig. 11 – Main features of Itch substrates *(from Melino et al., 2008, modified)*

Highlights on Itch substrates

Notch receptors

Notch has been proposed as one of the most relevant Itch target, and guessed to be responsible for the autoimmune phenotype of *Itchy* mice. The Notch pathway is a conserved signal transduction cascade which is essential for a wide array of cell-fate decisions and tissue-dependent differentiation and morphogenesis, including the induction of T and B cells and specification of T-cell effector fates, as T_H2 differentiation or activation and survival of mature peripheral T lymphocyte by upregulating Bcl-2 and IAPs (Jenkinson et al., 2006). During signal transduction cascade, Notch proteins undergo cleavage stimulated by the binding to an extracellular ligand or the activation of CD4+ lymphocytes, that finally release the intracellular domain portion (ICD). Itch-mediated polyubiquitylation of the ICD

Notch1 results in the degradation of the ICD, followed by receptor activation and hence modulation of the signal transduction. Interestingly, this regulation can be further tuned by the action of Numb, a protein which promotes the Itch ubiquitination of membrane-bound Notch1 receptor (see above).

Jun family members

As it could be easily guessed by *Itchy* mice phenotype, several Itch substrates should have a pivotal role in immune response. First lightings about the basis of immunological phenotypes show by these mice have came out from the identification of transcription factors c-Jun and JunB as Itch substrates. They both contain PY or PXY motifs that binds Itch WW domains. For JunB, Itch-mediated ubiquitylation drives to both lysosomal and proteasomal pathways; as a result, in Itch KO mice the expression of the JunB target gene *IL-4* (codify interleukin-4) is downregulated, attenuating the T_H2 cells response. Concomitantly the aberrant expression of JunB observed in *Itchy* mice drives T-cell differentiation towards T_H2 phenotype with hyperproliferation and abnormal allergic response (Fang et al., 2002).

c-FLIP

The proinflammatory cytokine tumor necrosis factor alpha (TNFα) is a signal both for cell survival (through IκB kinase IKK dependent activation of transcription factor NF-κB) and for apoptotic cell death (through the formation of a complex that contains the adaptor protein FADD and pro-caspase-8). NF-κB prevents caspase−8 activation through induction of cellular FLICE-inhibitory protein (c-FLIP, present in two isoforms, the longer c-$FLIP_L$ and c-$FLIP_S$), an adaptor protein which specifically inhibits caspase-8. Thus, NF-κB may negatively regulate TNFα-induced apoptosis through regulation of c-FLIP levels (reviewed in Wajant et al., 2003; Karin & Lin, 2002). On the contrary, JNK activity controls TNFα-induced death through the proteasomal degradation of c-$FLIP_L$ through activation of Itch, whose catalytic activity was just shown to be phosphorylation-dependent (see above).

p53 family members

Itch has also been shown to bind to, ubiquitylate and thereby negatively regulate p53 family members p63 and p73, but not p53.

Both p63 and p73 possess a canonical PY motif (while p53 does not) located in their C-terminal which accounts for the binding to Itch through its WW domains. It has been described that, under normal conditions, p63 and p73 protein levels are generally maintained low through Itch-mediated ubiquitylation. In response to genotoxic stress, p63 and p73 induction and and transcriptional activation are mainly controlled at post-translational level, and, at least partially, promoted by DNA-damage-caused Itch downregulation, whose molecular mechanism await further investigation (Rossi et al., 2005: Rossi et al., 2006).

1.1.5 RING E3s

The RING family of ubiquitin ligases is characterized by the RING domain which consists of a short cysteine/histidine rich motif (You & Pickart, 2001). The RING domain is conserved among species not in primary sequence but in spacing between residues, that coordinate two zinc ions. This motif is constituted by eight conserved cysteine and histidine residues (**Fig. 12**), and can be found in hundreds of different eukaryotic proteins. Two subtypes of RING motis can be identified: the RING-HC, carrying seven cysteines and one histidine, and RING-H2, which possesses six cysteines and two histidines. Several other proteins own RING domain (most of those shows ubiquitin ligase activity, as Mdm2 and Brca1), but not all RING domain proteins are E3 enzymes (Borden, 2000).

By a biochemical point of view, since RING domain cannot come closer than ~15Å to the E2 active site cysteine, it is easily to conclude that principal role of an E3 RING ligase is increasing the probability of reaction between the substrate lysine and E2-Ub complex by proximity, even if it is still puzzling how the substrate lysines can be oriented in a favourable position for reaction. In fact, most substrates appear to be ubiquitylated by RINGs on several lysines, and there is no consensus site for ubiquitination (despite of sumoylation) (reviewed in Pickart, 2001).

RING ligases can also be classified as either single or multisubunit ligases. For example, Mdm2 is a monomeric RING finger E3 for p53, which has an amino-terminal p53 binding domain

and a carboxyl-terminal RING finger and which is sufficient to ubiquitylate p53 together with E1 and E2 *in vitro* (Fang et al., 2000). On the contrary, dedicated multi-subunit E3s also exist in cells, including the SCF (**S**kp1-**C**ullin-**F**-box acronym), APC (**A**naphase **P**romoting **C**omplex)/Cyclosome, and CBC (elongin **C**-elongin **B**-**C**ul2). These contain a RING finger subunit and a member of the cullin family that binds the RING finger protein (see below). They also include structural adaptors that link the cullin to substrate recognition elements.

In this thesis work, I will focus on the superfamily of cullin-based E3 ubiquitin ligases, that belong to RING family, and that are discussed below.

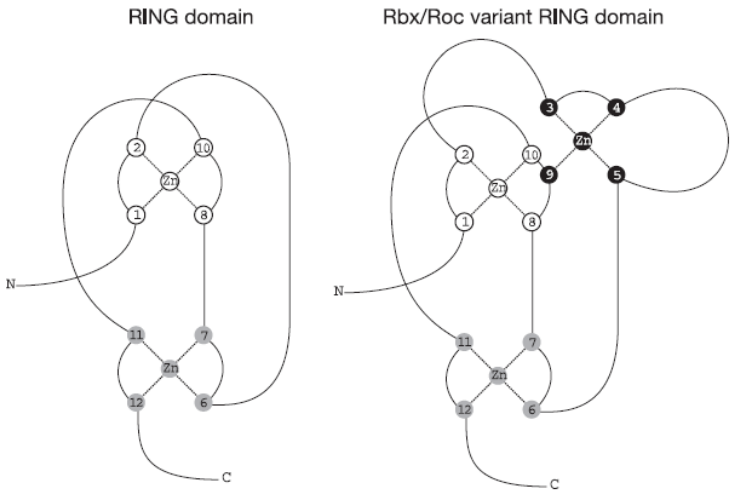


Fig. 12 - Schematic representation of the RING domain cross brace structure. They are shown both Zinc ions and the conserved residues that coordinate them *(from Willems et al., 2004)*

Overview of the cullin-based E3 ligases

The cullin–RING based ubiquitin ligases superfamily of enzymes controls a broad spectrum of cellular processes by coupling

the destruction of regulatory proteins to intracellular and extracellular signals. The specificity of these ligases, which share a modular organization, can be tuned by variable receptor subunits that recruit substrates to the cullin–RING catalytic core, leading to the possibility to potentially assemble hundreds of different complexes.

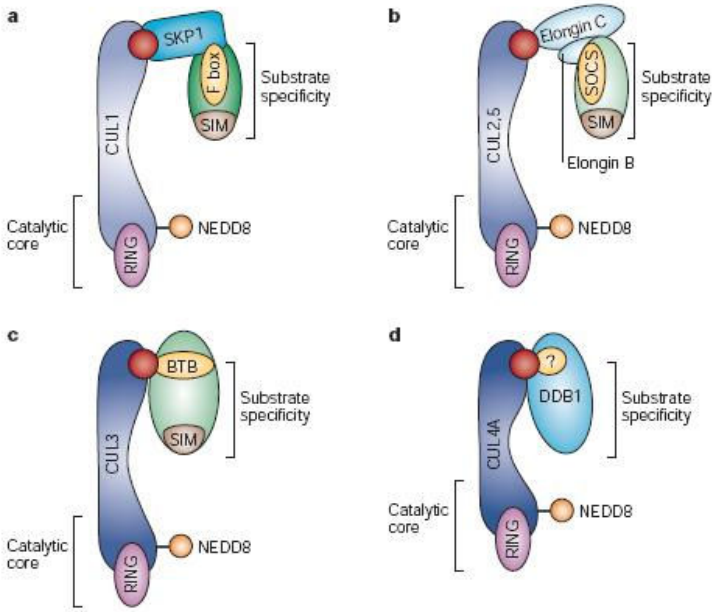


Fig. 13 – The superfamily of Cullin-based ubiquitin ligases. A. SCF complexes, that recruit substrates through the adaptor protein SKP1 and an F-box-protein substrate receptor. **B.** Cul2- and Cul5-based complexes recruit substrates through an elongin-BC adaptor and SOCS/BC-box-protein substrate receptor **C.** Cul3- based recruit substrates through BTB-domain-containing substrate receptor proteins. **D.** Cul4A based might recruit substrates through the adaptor protein DNA damage-binding protein-1 (DDB1), and through putative substrate-receptor complexes such as DET1–COP1. *(Petroski & Deshaies, 2005, modified)*

Human cells own seven different cullin proteins (Cul1, 2, 3, 4A, 4B, 5 and 7), each one part of a different multisubunit ubiquitin ligase, although other two proteins (the Apc2 subunit of the APC/C and the p53 cytoplasmic anchor protein Parc) could be add to the list, given that they contain a domain structural close to cullins (Yu et al., 1998; Nikolaev et al., 2003) (**Fig. 13**). Maybe the most famous and studied cullin-based multisubunit complexes are those that includes Cul1, named SCF complexes (SCFs), whereas the other that contain other cullins have been referred to by various names. SCFs represent the RING E3 ligases archetype (**Fig. 12**). It has been discussed above that, unlike the HECT ligases, cullin-based ones promote ubiquitylation without forming a covalent intermediate with ubiquitin; moreover, they can activate isopeptide-bond formation by at least four different E2 enzymes. Mammalian SCF and other cullin-based can work with either UBC3 or E2s of the UBC4/5 family *in vitro* (Strack et al., 2000). In addition, they recruits UBC12, which conjugates the ubiquitin-like protein NEDD8 to cullins (Kamura et al., 1998). It is worth to emphasize the importance of neddylation of cullins, which might control the dynamic equilibrium of complexes assembly and disassembly (see below).

The SCF E3 ubiquitin ligase family was discovered in budding yeast through cell cycle progression studies (Feldman et al., 1997). First insights into SCF-dependent proteolysis came out from some budding yeast *S. cerevisiae* cdc (**C**ell **D**ivision **C**ycle) mutants *cdc4*, *cdc34* and *cdc53* analysis (Willems et al., 1996). These mutants show cell cycle arrest before entry into S phase because they fail to degrade Sic1, which is the inhibitor of Cdc28 cyclin-dependent kinase (Schwob et al., 1994). Sic1 degradation depends on its phosphorylation by (Cln1/2)-Cdc28 complexes in G1 phase (Verma et al., 1997a). Afterwards it was found that Cdc53, Cdc4 and other two proteins, Skp1 and Rbx1 (alias Roc1), are part of a complex which shows E3 ligase activity. This complex acts in concert with the E2 enzyme Cdc34 to regulate G1/S transition through the degradation of phospho-Sic1 (Mathias et al., 1996; Feldman et al., 1997). In addition, G1 cyclins themselves are degraded in a Cdc34 and phosphorylation-dependent manner. Cdc53 is conserved through evolution: in *C. elegans*, the Cdc53 ortholog CUL-1 is also involved in cell division regulation, suggesting the

existence of a protein family conserved during evolution in their structure as well as in their function. The human ortholog of Cdc53 is Cul1.

The SCF model has been extended to the Cullin/Ring-based ligases superfamily, found through all eukaryotes. These enzymes regulate an impressive number of cellular processes far away from each other, such as DNA replication and circadian rhythms.

SCF complex structure and functions

In mammals, SCF complexes are composed by Skp1, Cul1 and Roc1 (also named Rbx1, the zinc-binding RING-H2 domain protein). Cul1 works as a scaffold, being able to bind both Roc1, which recruits the E2 enzyme, and Skp1, which acts as an adaptor for the fourth part of the complex, represented by proteins encharged to specific substrates recruiting. On the contrary, the N termini of other cullins recruit substrates by binding other proteins (**Fig. 13**). The highly conserved C-terminal domains of cullins bind to the RING subunit Roc1, except for Cul5, which seems to prefer Roc2 protein (Ohta et al., 1999). Cul1 structure is characterized by having a rigid and curved N-terminal stalk composed of three repeats of a five-helix bundle (cullin repeat, CR), linked to a C-terminal globular domain. Skp1 binds to the first CR region, whereas Roc1 binds tightly close to the C-terminal globular domain, 100 Å away from Skp1 (**Fig. 14**). The part of Cul1 that contacts Skp1 is highly conserved among its orthologues, but not among cullin proteins, which explains the reason why each cullin specifically recruits different set of adaptors, even if those used by other cullins show considerable structural homology to Skp1. The stalk of Cul1 might juxtapose the E2 and the substrate to promote ubiquitin transfer, given that a mutation that increases the flexibility of the stalk destroyed SCF activity *in vitro* (Zheng et al., 2002b).

As previously mentioned, specific substrates are recruited by proteins that contact the adaptor protein Skp1. Skp1 possesses two domains: an N-terminal segment that binds Cul1, and a C-terminal region that binds a ~40 amino acids motif called F-Box domain (Bai et al., 1996; Schulman et al., 2000). The proteins that own this domain are collected on a substrate-binding family named F-box family; they contain an N-terminal domain that binds Skp1

and a C-terminal region that binds substrate. Summarizing, F-boxes bind Skp1,which in turn binds Cul1, although the 3D structure of SCF^{SKP2} also revealed specific contacts between Cul1 and the F-box Skp2 (Bai et al., 1996). Therefore the number of F-box, and the other substrate-binding proteins that connect substrates to cullin-based complexes, represents the potential number of cullin-based ligases, although it is not known if all these can function as receptor subunits for cullins. In fact, it has been shown that at least two *S. cerevisiae* F-box proteins are not recruited into SCF complexes (Seol et al., 2001).

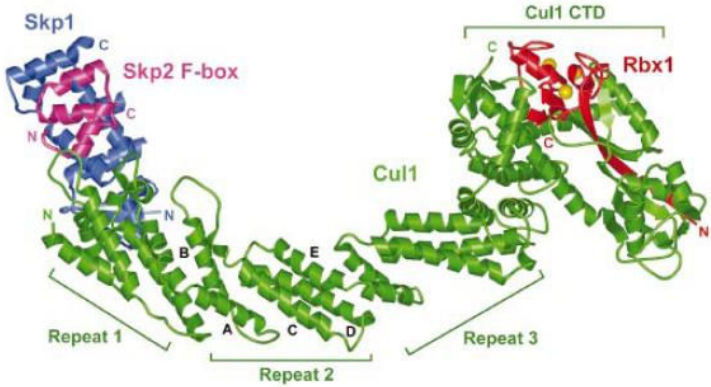


Fig. 14 - Overall structure of the SCF^{Skp2} complex. Cul1, Rbx1, Skp1 and the F-box Skp2 are coloured in green, red, blue and magenta, respectively. *(from Zheng et al., 2002b)*

Firstly, Skp1 was identified to form a complex in association with S-Cdk2 cyclin and another protein, Skp2, (Zhang et al., 1995); Cdc4 and Ctf13 (in *S. cerevisiae*) and Cyclin F (in *H. sapiens*) were then identified as interactors of Skp1 (Russel et al., 1999; Bai et al., 1996). All these proteins own a shared motif named F-box (from cyclin F), which is the binding site for Skp1. The observation that Cdc53, Cdc34 and Skp1 are common components in the degradation of Sic1 and Cln2 (G1 cyclin), while Cdc4 and Grr1 are divergent, was typify as the "F-box hypotesis", which

enunciates that proteins containing the F-box motif could both bind the complex Skp1-Cdc53-Rbx1 through direct interaction with Skp1 and recruit substrates for ubiquitination through their specific protein-protein interaction domains, such as the WD-40 or leucine-rich repeats (LRR), giving specificity to the core complex (Bai et al., 1996). Eleven in yeast, 326 in *C. elegans*, 22 in *D. Melanogaster* F-boxes have been found up to date, and in *H. sapiens* approximately 70 genes codify these proteins (Kipreos & Pagano, 2000; Yoshida, 2007). The widespread number of F-box proteins reinforces the F-box hypothesis with evidences they have a pivotal role in regulatory and signalling pathways, specially given that the main part of SCF complexes substrates must be phosphorylated to be recognized (Skowyra et al., 1997).

Another interesting observation on SCF complexes activity came out from budding yeast and regards regulation of SCF complexes activity. The conserved ubiquitin-related protein called Rub1 in yeast and plants, and known as Nedd8 in metazoan, was found to be covalently bound to a conserved lysine residue in C-terminal region of almost all cullins. Cullins must be modified by the covalent attachment of Nedd8 to a conserved Lys in order to have an active SCF complex (Hori et al., 1999; Osaka et al., 2000). Neddylation enhances Cul1-dependent ubiquitin-ligase activity *in vitro* (Wu et al., 2000). *In vivo*, a fraction of the total pool of a cullin protein is neddylated, which indicates that perhaps only a subsetof cullin molecules are fully active. Nedd8 conjugated to cullins is detached by the COP9 signalosome (CSN) (Cope et al., 2002). This activity regulation mechanism is more finely tuned: it has been shown that a Cul1-binding protein, Cand1, competes with Skp1 for binding to Cul1 for only to Cul1 molecules that are not conjugated to Nedd8 (Zheng et al., 2002a) (**Fig. 15**). Although the structures of a number of CRL complexes have been solved (Zheng et al., 2002b, Goldenberg et al., 2004, Angers et al., 2006), the mechanism by which Ub is transferred from the E2 to the substrate remains elusive. Recently reported evidences shed new light on the structural basis underlying this final step of ubiquitin transfer.

The available models of CRL show a perplexing special gap between the substrate receptor and the E2 docked to the Roc1. Furthermore, with the addition of each Ub molecule, the distance between the distal end of the growing poly-Ub chain and the E2 active site is expected to change. How the E3 machinery facilitates

substrate ubiquitination across this large and ever-changing gap? The answer to this question has come from an understanding of how CRLs are activated by the Ub-like protein Nedd8 (Duda et al., 2008; Saha & Deshaies, 2008). Neddylation seems to have a broader range of effects on SCF, as helping recruit E2 to Roc1, bringing the E2 in closer proximity to the substrate, stabilizing the transition state of the growing poly-Ub chain at the E2 active site.

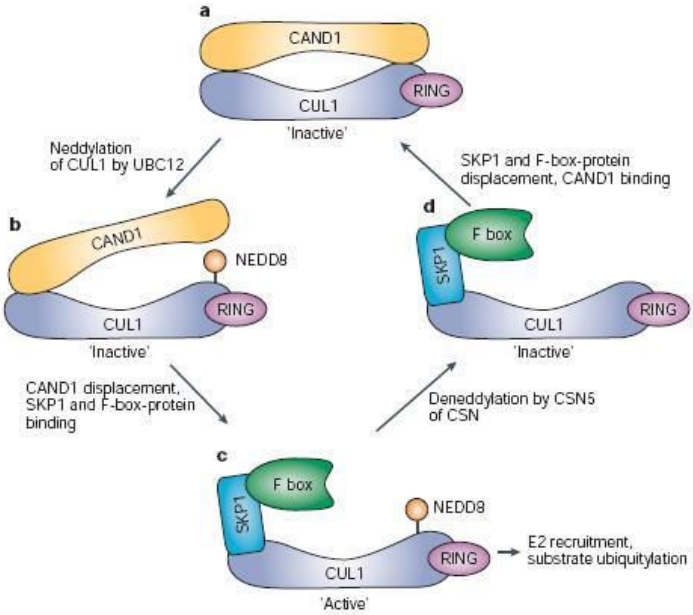


Fig. 15 - The regulation of cullin–RING-ligase activity through the Cand1/Nedd8 cycle. A. When SCF is assembled with Cand1, it is held in an inactive state. **B.** Neddylation of cullin results in CAND1 dissociation. **C.** This might enable the assembly of a SKP1–F-box, allowing ubiquitylation of substrates. **D.** Nedd8 might be detached from cullin through the isopeptidase activity of the CSN5 subunit of CSN, which, in turn, might lead to the dissociation of Skp1 and the sequestration of Cul1 by Cand1 *(from Petroski & Deshaies, 2005, modified)*.

In *S. cerevisiae*, Rub1 conjugation requires at least three proteins *in vivo*: Ula1 and Uba3, related to the N- and C-terminal domains of the E1 ubiquitin-activating enzyme respectively, and Ubc12, a protein related to E2 ubiquitin-conjugating enzymes, which functions analogously to E2 enzymes in Rub1-protein conjugate formation (Lammer et al., 1998; Liakopoulos et al., 1998). In humans, neddylation of cullins is catalyzed by the bipartite activating enzyme APPBP1-hUba3 and a dedicated E2 enzymes called Ubc12, even if the specific E3 has not yet be found (Osaka et al., 1998). The biological significance of this modification appears to reside in facilitation of E2 recruitment, in strengthening of substrate ubiquitination efficiency and in the regulation of the complex assembly (Kawakami et al., 2001; Furukawa et al., 2000).

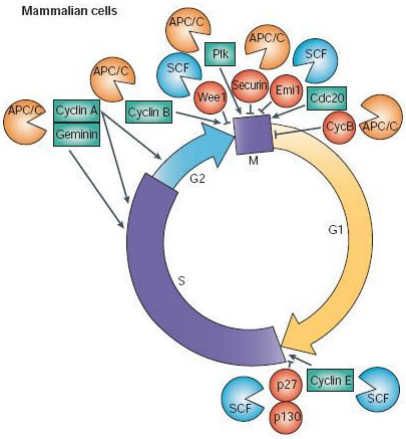


Fig. 16 - Key cell-cycle regulatory proteins targeted by ubiquitin-mediated proteolysis *(from Reed, 2003, modified)*

Finally, it must be sketched that some cullin-based ligases seem to mix components from other ubiquitin-ligase pathways. For example, an SCF-like complex identified in neuronal cells of *C. elegans* can recruit Rpm-1 (a large protein with numerous RING domains) instead of Roc-1, in a complex which contains SKP1,CUL-1 and the F-box-protein FSN-1. This complex regulates the stability of proteins that are involved in presynaptic differentiation (Liao et al., 2004).

In almost every examined case, substrates of cullin-based ligases are covalently modified. Phosphorylation targets numerous substrates to SCF ligases, e.g. the cyclin-dependent kinase (CDK) inhibitor Sic1 to SCF^{Cdc4} in *S. cerevisiae* (Verma et al., 1997a; Verma et al., 1997b, see above) and cyclin E to SCF^{CDC4} in humans (Clurman et al., 1996). Phosphorylation seems to be the predominant signal, but not the only one (e.g. Jaakkola et al., 2001). Phosphorylation is one of the major mechanisms used by cells to rapidly trasduce signals. Hence a connection with SCF complexes seems ideal to act in concert with such dynamic processes that require an abrupt change to be at short or long term irreversible, through the degradation of key proteins. Good examples of this coordinate action are the roles exerted by SCF complex in cell cycle phase transitions, via the degradation of cell cyle regulators (reviewed in Deshaies, 1999; Reed, 2003) (**Fig. 16**).

A hitchhiker's small guide to F-boxes proteins

The F-box domain is a protein motif of approximately 40 amino acids that functions as a site of protein-protein interaction. The F-box motif links the F-box protein to other components of the SCF complex by binding the adaptor protein Skp1. F-box proteins (FBPs) often include additional carboxy-terminal motifs capable of protein-protein interaction: the most common of which are WD repeats and leucine-rich repeats in yeast and humans (see below), both found to bind phosphorylated substrates to the SCF complex, even if the majority of FBPs have other associated motifs. In addition, FBPs have recently been discovered to function in non-SCF protein complexes and in a variety of cellular functions. The number of predicted and described FBPs is growing and growing, but the functions of most of these proteins have not yet been defined.

F-box proteins are protagonist in several cellular scenarios including cell cycle progression, synapse formation, plant hormone responses, the circadian clock and DNA damage response. As mentioned before, in addition to the shared F-box domains at the N terminus, FBPs often carry other domains such as WD40 repeats (WD40) or leucine-rich repeats (LRRs) at their C terminus for substrate recognition. WD40 repeats display β-propeller structures, and LRR repeats are arc-shaped a-b repeats that mediate protein-

protein interaction (reviewed in Kobe & Kajava, 2001). The FBPs that own WD40 repeats are classified as "FBXW" (where "W" means WD40), while the ones that include LRR repeates are called "FBXL" ("L" is for LRR repeats). Some other domains such as Kelch repeats, carbohydrate-interacting (CASH), and proline-rich domains are also present at the C terminus of FBPs, named "FBXO" (with the "O" for Other domains). While the expression of mRNAs encoding some FBPs have been found in all tested tissues, other ones are clearly tissue-specific (Cenciarelli et al., 1999). Regarding FBPs localization within the cell, only a few studies have been performed, and almost in all it have been used overexpressed tagged FBPs because of the lack of commercial antibodies. Some FBPs were found both in the cytoplasm and in the nucleus, and the comparison between the localization of wild-type and F-box domain lacking mutant revealed that the ability to bind Skp1 doesn't determine their subcellular localization (Cenciarelli et al., 1999).

The three most famous and studied FBPs are Skp2, Fbw2 and β-TrCP. These FBPs are well characterized and extensively studied because of their relationship with cancer. Indeed, they exert a pivotal role in cell cycle regulation, targeting substrates that are involved in cell cycle arrest and promotion **(Fig. 17).** Skp2 promotes the cell cycle through the ubiquitylation of CDK inhibitors, including p27, and thereby promotes cell cycle progression during S and G2 phases (reviewed in Frescas & Pagano, 2008). Therefore it functions as an oncoprotein, in fact it is overexpressed in many cancers. By contrast, Fbw7 induces the degradation of positive regulators of the cell cycle, such as Myc, Jun, cyclin E and Notch. Fbw7 targets cell-cycle promoters to be degraded in order to inhibit the cell cycle, hence serves as a tumour suppressor. Mutations or deletions in Fbw7 are found in a subset of human cancers (reviewed in Welcker & Clurman, 2008). Finally, β-TRCP is a versatile F-box protein that recognizes several cell-cycle regulators, both cell-cycle promoters and inhibitors including Emi1/2, Wee1a, Cdc25A/B, but also β-catenin and IκB. Wee1a and Cdc25A are a kinase and a phosphatase that negatively and positively regulate CDK activity, respectively. Wee1a expression is decreased by ubiquitin-dependent degradation at the onset of M phase, and its degradation is required for the rapid activation of Cdk1; Cdc25A is phosphorylated by the checkpoint kinases Chk1 and Chk2 in response to DNA damage or stalled DNA replication. Phosphorylation of Cdc25A allows the

protein to be recognized by β-TrCP and ubiquitylated. These data indicate that β-TrCP has a prominent role in controlling the timing of entry into M phase, and in mediating the response to DNA damage. As easily guessed, genetic alteration of β-TrCP genes in human cancers have been found, both mutations or overexpression (reviewed in Frescas & Pagano, 2008).

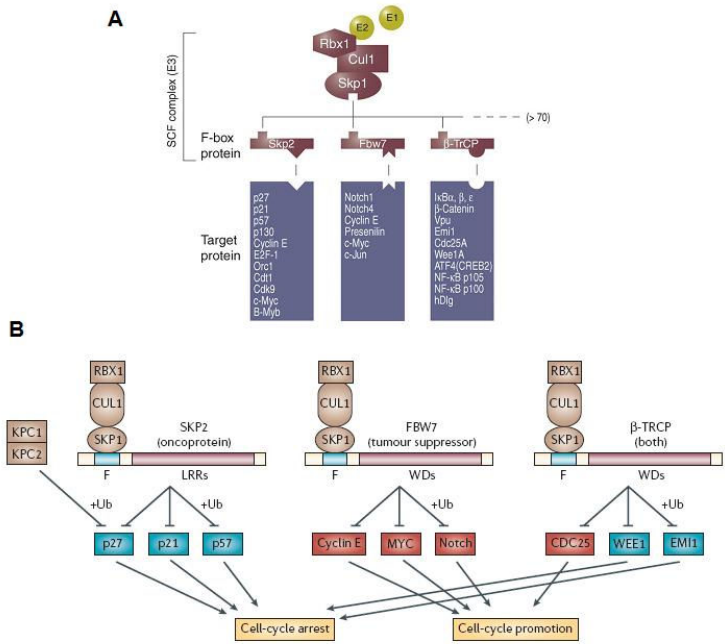


Fig. 17 - The SCF complexes containing Skp2, Fbw7, or β-TrCP target various substrates for ubiquitylation. A. This schema summarizes the known substrates of the most studied FBPs Skp2, Fbw7 and β-TrCP. **B.** Among the many F-box proteins, Skp2, Fbw7 and β-TrCP have been shown to control the abundance of cell cycle regulators. *(from Nakayama KI & Nakayama K., 2005; 2006, modified)*

It has been discussed above that FBPs are the SCF complexes subunit known to provide the specificity regarding the

substrate. In the majority of the studied cases, substrate phosphorylation is one common prerequisite for FBPs recognition: one or more residues of the substrate constitute the sequence, called "phosphodegron", with which FBPs specifically interact with. In mammals, well-knows examples of phosphodegrons are represented by the DpSGFXpS motif (in which F is a hydrophobic amino acid and pS is a phosphoserine), which is present in all the known substrate of the F-box protein β-TrCP. Another well characterized phsphodegron is recognized by the F-box protein Fbw7, and it is characterized by a phosphorylated serine at the +4 position after a threonine.

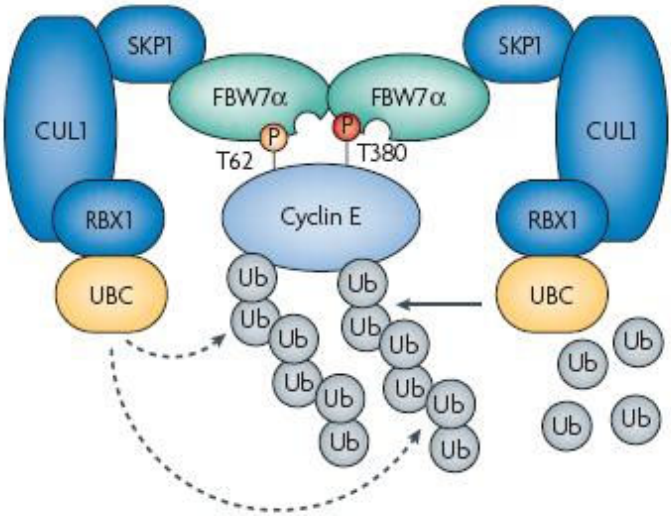


Fig. 18 - Speculative model for FBW7 dimerization. Proposed model in which the Fbw7 dimer contacts simultaneously both phosphodegrons present on its substrate Cyclin E, thereby stabilizing the interaction with low-affinity degrons. (*from Welcker & Clurman, 2008).*

Some FBPs are proposed to act as dimers, and dimerization has been proposed to be a general phenomenon conditionally required for their target recognition **(Fig. 18)**. For example, mammalian FBPs β-TrCP1 and β-TrCP2 have been described to form homo- or heterodimers that regulate IkBα, although only homodimers of each participate in the ubiquitination and degradation of it (Suzuki et al., 2000). Instead, Fbw7 contains three differentially spliced isoforms: α, β, and γ that form homo- or heterodimers. Since these isoforms reside in different subcellular compartments, it is has been speculated that heterodimer formation alters the original localization of single isoforms (Welcker & Clurman, 2007). Dimerization has also been observed for the mammalian FBP Skp2, the fission yeast FBPs Pop1p, and Pop2p.

Dimerization of F box proteins is directed through the so-called D domain. The D domain is localized at N-terminal to the F box region, and critical hydrophobic residues (leucine and isoleucine) are conserved spatially in distinct F box proteins such as Fbw7, β-Trcp, and Fbw2 (Zhang & Koepp, 2006). Although this phenomenon is established, the functional significance of this and whether the dimerization is constitutive or regulated remain to be elucidated.

Some FBPs also act independently, in an SCF-free context. In fact, several evidences have indicated that some FBP possess a non-degradative function, although still able to bind to Skp1. For example, in budding yeast, three Skp1-containing, non SCF complexes were described: FBPs Mfb1 and Mdm30 regulate mitochondria fusion and tubule formation (Durr et al., 2006); FBP Ctf13 and Skp1 are cointained in CBF3 kinetochore-binding complex (Kitagawa et al., 1999); and, finally, FBP Fbh1 is a DNA-dependent ATPase with a DNA-unwinding helicase activity (Kim et al., 2002) **(Fig. 19)**.

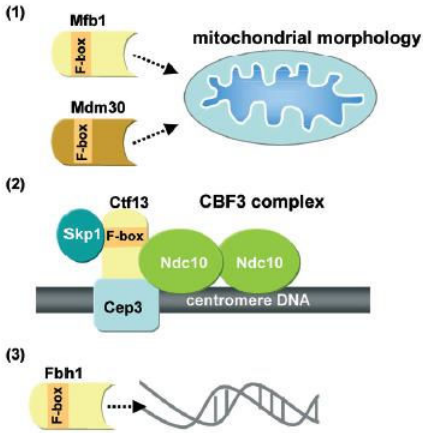


Fig. 19 - Three budding yeast FBPs that works independently of the SCF complex. *(from Ho et al., 2008, modified)*

In addition to ubiquitination, even neddylation was found to be regulated by the FBPs. In fact, by overexpression of both all the subunit of the SCF^{Skp2} and its substrate p27 in human cells, it has been found that Cul1 is increasingly neddylated, suggesting that the presence of the FBP and its substrate alters Cul1 neddylation levels; moreover, it overpowers the effects of Cand1 and prevents the deneddylation action performed by CSN (Bornstein et al., 2006). Cul1 is not the only example of FBPs-dependent neddylation: recently it has been reported that FBXO11, in concert with the other components of SCF complex, promotes the neddylation of p53 at two lysines within its nuclear localization signal. However the stability of p53 is not affected, hence it has been hypothesized that FBXO11 regulates p53 localization, thus affecting its transcription activity and suppressing the function of p53 (Abida et al., 2007).

1.2 The p53 family

As widely known, the tumor suppressor p53 is crucial in maintaining cellular genomic integrity and in controlling cell

growth. Loss or gain of p53 function results in the aberrant growth of cells (Levine, 1997). As easily guessed, both the cellular expression and the activity of p53 are tightly regulated. p53 protein has a very short half-life and thus is usually present at extremely low levels within cells. However in response to stress and DNA-damaging agents p53 is transiently stabilized and activated. Depending on cell type, cell environment and oncogenic alterations, this activation leads to inhibition of cell cycle progression, induction of senescence, or apoptosis (reviewed in Vousden & Lu, 2002; Vousden & Lane, 2007). In the late '90s, just over a decade after the identification of p53, two p53-related genes, p63 and p73, were identified (Kaghad et al., 1997; Jost et al., 1997; Yang et al., 1998). The p53, p63 and p73 genes are located on different chromosomes (17p13.1, 3q27–29 and 1q36, respectively), and all three genes are now known to express many differentially spliced isoforms.

p73 and p63 are modular proteins with a similar basic structure, which is schematically depicted in **Fig. 20**.

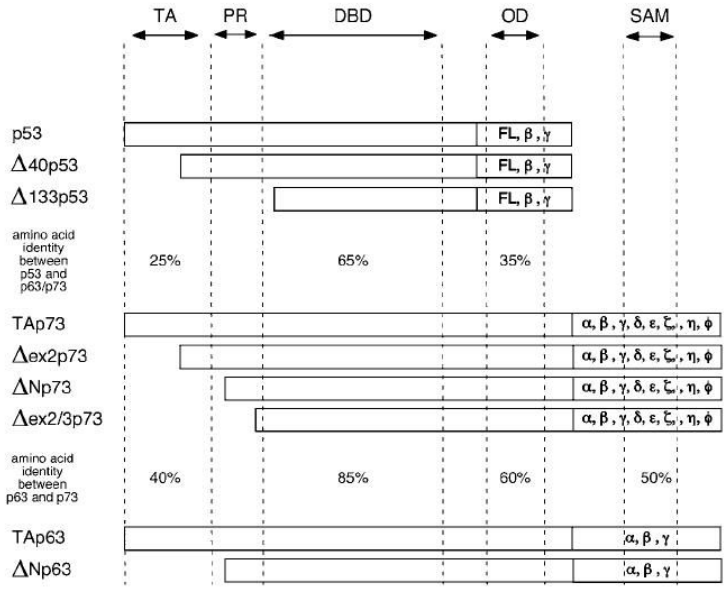


Fig. 20 - Schematic presentation of p53, p63 and p73 isoforms *(from Pietsch et al., 2008)*

Like p53, both p63 and p73 possess an N-terminal transactivation domain (TAD), a DNA binding domain (DBD), and a C-terminal oligomerization domain (OD) (Murray-Zmijewski et al., 2006). Although p63 and p73 demonstrate relatively little homology with p53 in their TAD and OD, both share approximately 60% similarity with the DBD of p53. In addition, the residues of p53 that directly interact with DNA are identical in p63 and p73; consequently, they both can bind to canonical p53 binding sites and activate transcription from p53-responsive promoters, and inducing cell cycle arrest or apoptosis. However, p73 and p63 display an higher level of structural complexity than p53, given that several isoforms that arise from alternative splicing at C-terminus or from using an additional promoter have been identified. In fact, the P1 promoter of both genes is embedded in a non-coding region of exon 1, while the P2 promoter is located in intron 3. The first promoter (P1) yields a full-length protein possessing a N-terminal TAD (TA isoforms), while the second one (P2) gives rise to N-terminally truncated proteins (ΔN isoforms). The ΔN isoforms can bind but not activate p53-responsive promoters, since they lack the TAD, acting thus as dominant negative inhibitors of p53 and the full-length TA variants (Grob et al., 2001; Stiewe et al., 2002). Carboxy-terminal isoforms are due to alternative splicing of exons 11, 12 and 13, which code for the sterile alpha motif domain (SAM). This domain is a protein-protein interaction domain composed of four α-helices and a small 3_{10}-helix. SAM domain is found in p73-related but also in several unrelated protein that are involved in regulation of development (Thanos & Bowie, 1999). SAM domain often mediate homodimerization, but not in the case of p73, in fact p73 can forms homodimers and heterodimers with other splicing variants and with wild-type and mutant p53, but in a SAM-independent manner (Arrowsmith, 1999).

Summarizing, for p73, eight different isoforms of p73 have been described: TA and ΔN p73α, β, γ, δ, ε, ζ, η and θ (**Fig. 20**). Sequence comparison between p53, p63 and p73 in different species clearly shows a p63/p73-like protogene as the ancestor of the family, even if p53 was the first one identified and the most studied (**Fig. 21**).

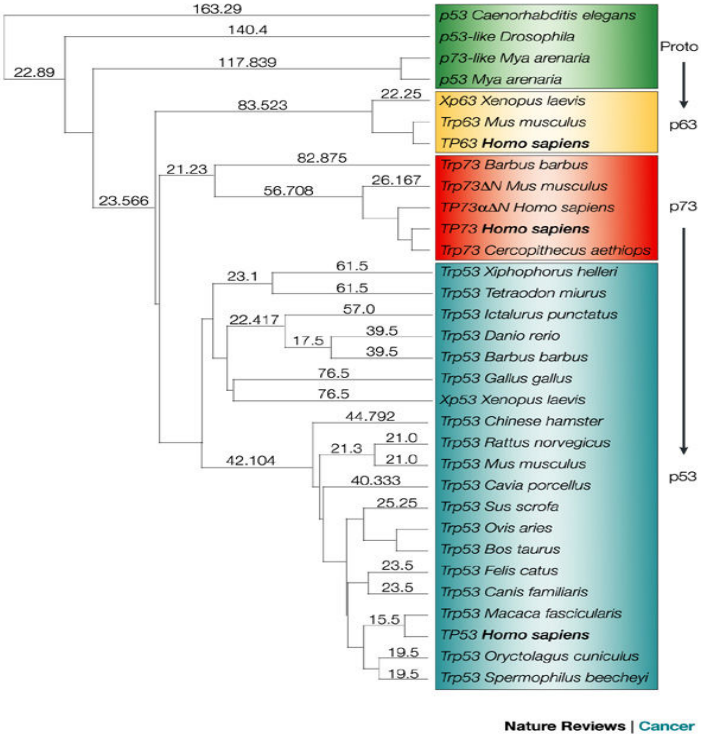


Fig. 21 – Evolution within the p53 family *(from Melino et al., 2002)*

1.2.2 p73: a transcription factor and much more

The *TP73* gene maps to a region (1p36.33) that is frequently deleted in neuroblastoma, indicating that loss of p73 function might have a role in this tumour development; however, mutation of *TP73* are rare in human cancer (reviewed in Melino et al., 2002; Moll & Slade, 2004). As previously introduced, TP73 encodes different isoforms that are expressed under the control of two different promoters. These isoforms act antitethetically: the transcriptionally active full-length TAp73 induces cell-cycle arrest and apoptosis, while the amino-terminal truncated ΔNp73 acts as dominant negative by inhibiting both TA- and p53 dependent

apoptosis (see below, and **Fig. 24**). Moreover, ΔNp73 expression is induced by TAp73 and p53, creating a negative feedback loop (Grob et al., 2001; Ishimoto et al., 2002). In other words, the ratio between the protein levels of TAp73 and ΔNp73 in cells can be a crucial factor to determine the cell fate.

Based on their structural similarity, p73 and p53 should exert similar outcomes. Similarly to p53, p73 is activated in response to a variety of genotoxic agents, including DNA-damaging agents and the oncogenes E1A and myc (Zaika et al., 2001). Once activated, p73 can regulate the induction of apoptosis and cell cycle arrest, and shares with p53 several target genes as p21, 14-3-3s, GADD45, BTG2, PIG3, p53R2 and IGFBP3 (**Fig. 22**; reviewed in Scoumanne et al., 2005). But differences in behavior between p53 and p73 are well known, and can be partly explained by evidences that p73 can transactivate specific genes, e.g. the cyclin-dependent kinase inhibitor p57 KIP2, activated by TAp73β but not by p53 (Blint et al., 2002). It is important to underline that TAp73 splicing variants differ in their ability to induce p53 target genes. In particular, TAp73β seems to be the more potent inducer than the other variants (Ueda et al., 1999). Other examples can be represented by evidences that TAp73β and TAp63, but not p53, enhance Wnt/β-catenin (Osada et al., 2005); and that also interleukin 4 alpha is selectively regulated by p73 in several human cancer cell lines (Sasaki et al., 2003). But *TP73* also gives rise to DN isoforms, and most reports suggest that they do not cause cell cycle arrest and apoptosis, given that ΔNp73 proteins cannot transactivate genes but, retaining their DBD and OD domains, can account for binding to target genes and oligomerization with the other members of family, so can thus act as dominant-negative inhibitors of both wild-type p53 and TAp73.

Tightly summarizing, the main opinion on p73 functions is that in a p53-null background TAp73 can be responsible for regulating of the cell cycle, apoptosis and cancer cell response to genotoxic stress. p73 activates many p53-related genes, and it can also induce activities that are not shared by p53 and that can even be oncogenic, as exemplified by β-catenin (see below).

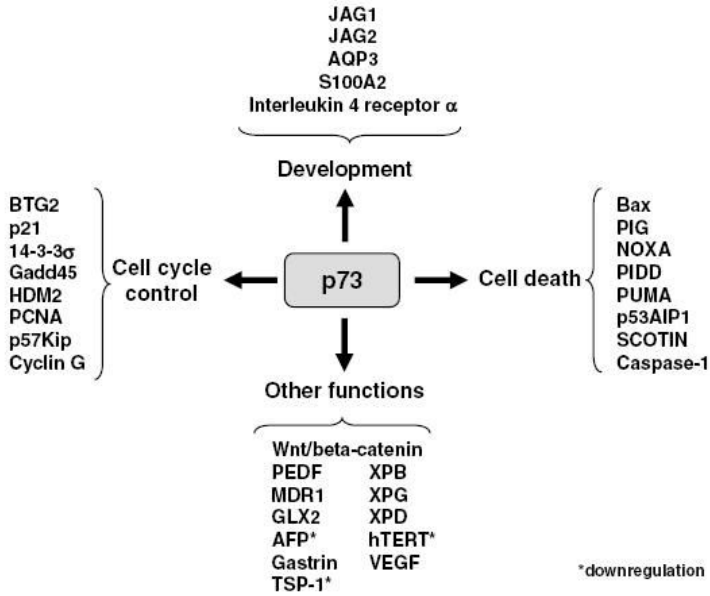


Fig. 22 - Downstream target genes directly regulated by p73 *(Marabese et al., 2007)*

Both transcription-dependent and independent p73 induced apoptosis have been described, even if p73-dependent apoptosis seems to be primarily regulated by its ability to transcriptionally activate pro-apoptotic p53 target genes, including the BCL2 family members BAX, PUMA, NOXA, BAD and BIK, the oxidoreductase PIG3, the tetraspan membrane protein PERP, the death receptors CD95, TNF-R1, TRAIL-R1 and TRAIL-R2 (TNF-related apoptosis inducing factors), the mitochondrial membrane protein p53AIP1, as well as the caspases-3, -6 and -8 (Melino et al., 2002; Müller et al., 2005). A transcription-independent role of p73 in apoptosis is disputed. It has described that upon DNA damage and TRAIL stimulation p73 can be cleaved by caspases-3 and -8, and both full-length and caspase-cleaved p73 localized to the mitochondria. Importantly, a transactivation deficient p73 mutant was found to enhance TRAIL induced apoptosis, and the addition of recombinant p73 to purified mitochondria resulted in cytochrome C release

(Sayan et al., 2008). However these findings are in contrast with an earlier publication that reported that although p73 induces BAX translocation from the cytosol to the mitochondria, p73 itself does not localize there, and that the translocation of BAX is not a direct effect of the interaction with p73 (Melino et al., 2004).

Despite the similarity between p53 and p73, they exhibit distinct biological functions, reflected by knockout mice. Unlike p53 null mice, which exhibit increased susceptibility to spontaneous and induced tumors but are viable and develop normally (Donehower et al., 1992), p63 and p73 knockout mice show mainly specific developmental defects, but do not develop spontaneous tumors. p63 knockout mice show severe developmental defects, including failure to develop limbs, skin and other epithelial tissue; these mice do not survive beyond a few days after birth (Keyes et al., 2006; Mills et al., 1999). p73 knockout mice exhibit neurodevelopmental (hippocampal dysgenesis due to inappropriate apoptosis of Cajal-Retzius cells and hydrocephalus) and inflammatory (chronic infections and excessive inflammation) defects (Yang et al., 2000). In these mice, the neuronal defects are a result of the absence of the anti-apoptotic ΔNp73 isoforms that normally block p53-mediated apoptosis in developing mouse neurons; in addition, they show runting and abnormal social and reproductive behavior due to defects in pheromone detection.

As show by these evidences, although it is clear that p73 is important for normal development, its role in tumor suppression is controversial. Most human tumors lack mutations or deletions of the p73 locus (reviewed in Deyoung & Ellisen, 2007). Also, many tumors upregulate ΔNp73, often in coincidence with TAp73 (Concin et al., 2004; Stiewe et al., 2004). Interestingly, animals heterozygous for p63 or p73, as well as both double heterozygous p53/p63 or p53/p73 mice, showed no significant differences in tumor latency, spectrum or frequency after gamma-radiation in p53-mediated mouse lymphoma, compared to their control counterparts (Perez-Losada et al., 2005). But recently, re-analysis of aging p53+/−p73+/− mice revealed an elevated incidence of microscopic carcinomas, T lymphomas and sarcomas, and increased metastatic ability, compared to p53+/− mice (Flores et al., 2005), profiling a conflicting role for p73 in tumor development, leading to investigate the relationship between the loss of genomic integrity and p73 function in cancer development by using mouse embryo fibroblasts

proficient or deficient for p73 in p53 +/+ or -/- context. Isolated loss of p73 disrupts normal cell-cycle regulation, and impairs proliferation and transformation due to compensatory p53 activation. Although co-deleting p53 rescues these defects, combined loss of p73 in a p53-deficient background strikes the genomic stability of cells, resulting in polyploidy and/or aneuploidy, more than loss of p53 alone (Talos et al., 2007). All these data indicate a conflicting role for p73 in tumor development, probably due to the pro- and antiapoptotic functions exerted by TAp73 and ΔNp73, respectively. To determine which aspects of p73 function are attributable to the TAp73 isoforms, more recently it has been generated and characterized TAp73 isoforms specifically deleted mice that develop a phenotype intermediate between the phenotypes of Trp73(-/-) and Trp53(-/-) mice with respect to incidence of spontaneous and carcinogen-induced tumors, infertility, and aging, as well as hippocampal dysgenesis (Tomasini et al., 2008).

Many studied have been addressed to investigate mutations in the *TP73* gene in tumorigenesis, taking advantage from the widely confirmed evidence that p53 is very often mutated in human tumours, To date, *TP73* is described to be very rarely mutated (fewer than 0.5% of human cancers), Contrarily, p73 was reported to be overexpressed in many tumours such as ependymoma, breast, neuroblastoma, lung, esophagus, stomach, colon, bladder, ovary, hepatocellular carcinoma, myeloid leukaemia, but when these studies were performed, ΔN isoforms were not yet discovered, hence the observed hyperexpression summarized the overall expression of both TA and ΔNp73 mRNAs, given that quantitative reverse transcription-polymerase chain reaction methods were mainly used (Zaika et la., 2001). When specific primers for TAp73 or ΔNp73 were used to measure p73 levels in tumours, it could be established that dominant negative ΔNp73 isoforms rather than TAp73 might be physiologically more important tumours overexpression (Concin et al., 2004). But how can ΔNp73 isoforms exert a dominant-negative effect on p53 and TAp73? This inhibitory function can be carried either at the oligomerization level, or by competing for binding to the same DNA target sequence (**Fig. 23**; Stiewe et al., 2002).

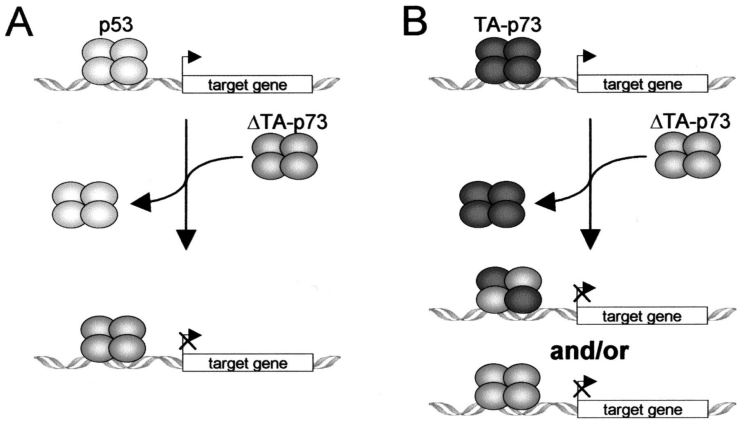


Fig. 23 - Model for the dominant-negative mechanism of ΔNp73 *(Stiewe et al., 2002)*

Moreover, the ΔNp73 promoter contains a very efficient p53/ TAp73 responsive element, hence p53 and TAp73 can induce expression of ΔNp73, and therefore create a dominant-negative feedback loop that regulates the function of both p53 and TAp73 (Grob et al., 2001). similarly to the Mdm2 loop (reviewed in Levine, 1997), given that both Mdm2 and ΔNp73 are direct transcriptional targets of p53 and both inhibit p53 function (**Fig. 24**).

Anyway the reason why TAp73 or ΔNp73 could be overexpressed is still unclear. It has been speculated that it could be related to a disfunction in transcriptional activity of P1 and P2 promoters or to a longer stability of their mRNAs. But it could be also guessed that the steady state proteins levels could be affected by changes in the many post-translational modifications described for p73 (see below).

In the context of the study of relationship between p73 and tumorigenesis, the p73 P1 promoter has been cloned and studied. Several E2F potential binding sites were identified, maybe exerting a role in p73 transcriptional regulation. E2F-1 directly activates transcription of p73 which leads to the activation of p53-responsive target genes and apoptosis. E2F-1 belongs to a family of transcription factors that are key regulators of cell cycle, DNA damage repair and apoptosis, so the E2F-1-dependent activation of

p73 represents a way to induce death in the absence of p53. (Irwin et al., 2000). Obviously, several other transcriptional factors may be involved in p73 regulation; for example, the P1 promoter can be regulated by p53 through the p53 potential binding site, even if it is not responsible for the DNA damage-dependent activation of p73 (Chen et al., 2001; Marabese et al., 2003).

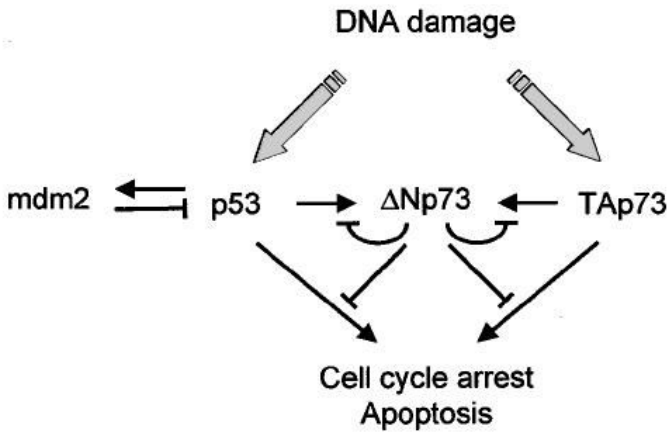


Fig. 24 - Schematic representation of the p53 loop *(Grob et al., 2001)*

As previously depicted, another important evidence came out from the study of the p73 promoters: after cloning the P2 promoter it was observed that the expression of ΔNp73 was strongly up-regulated by the TA isoforms and by p53, creating a feedback loop that tightly regulates the function of both TAp73 and p53, because of a a p53-responsive element in the p73 P2 promoter. So the expression of ΔNp73 not only regulates the function of p53 and TAp73 but also shuts off its own expression (Grob et al., 2001).

1.2.3 Post-translational modifications underlying p73 protein stabilization and transcriptional activation

As for p53, p73 protein levels are maintained at low levels, and its activation is mainly controlled at posttranslational level. Several modifications have been described, including phosphorylation by c-abl (Agami et al., 1999; Yuan et al., 1999; Gong et al., 1999) and p38MAPK (Bernassola et al., 2004), acetylation by CBP/p300 (Costanzo et al., 2002), SUMOylation (Minty et al., 2000), and c-Jun activation (Toh et al., 2004).

Both transcriptional activity and protein stability of p73 has been shown to be affected by DNA damage. The MLH-1/c-abl signalling induces stabilization of p73 through its phosphorylation on Tyr residue99 (Agami et al., 1999), and indirectly through the activation of the p38 MAP kinase pathway that phosphorylates p73 on Ser/Thr-Pro residues (Sanchez-Prieto et al., 2002). However DNA-damage dependent induction of p73 can also be trigged off through p300-mediated acetylation (Costanzo et al., 2002), or through tumor suppressor promyelocytic leukemia protein (PML) which binds p38MAPK-phosphorylated p73 and recruited it into PML-nuclear bodies (PML-NB), resulting in p73 protein stabilization (Bernassola et al., 2004). moreover, it has been described that DNA damage-induced phosphorylation of p73 on Ser/Thr-Pro residues allows the interaction of p73 with the peptidyl–prolyl cis/trans isomerase Pin1, resulting in stabilization of p73 protein and enhancing the transactivation and apoptotic activity of p73 (Mantovani et al., 2004). Pin1 and p73 interact in a c-Abl-dependent manner and this interaction requires phosphorylation of p73 on tyr99. Moreover, Pin1 augments binding of p73–p300 and stimulates subsequent acetylation of p73 by p300.

In 2003, a study reported that p73a is a target of checkpoint kinase 1 (Chk1), an essential component of the DNA damage response. Upon DNA damage, Chk1 interacts with and phosphorylates it on serine 47 endogenous p73α, activating the apoptosis-inducing functions of p73 (Gonzales et al., 2003). In 2005, our laboratory has demonstrated that Itch, a Nedd4-like HECT-E3 ubiquitin ligase, targets p73 for protein ubiquitination (Rossi et al., 2005). Though both Nedd4 and Itch physically interact with p73, only Itch is able to ubiquitinate and degrade both TA and ΔNp73. p73 binds to the WW domains of Itch through its PY motif,

localized at C-terminus of p73. As a result, Itch has no effect on the shortest p73 proteins, such as the γ and δ isoforms, and on p53, given that these proteins do not contain a PY motif. The overexpression of Itch stimulates the ubiquitylation of p73 in vivo, thus inducing a rapid proteasome-dependent degradation of p73. Conversely, the siRNA-mediated silencing, or the genetic ablation of Itch results in accumulation of p73. Importantly, upon DNA damage, Itch protein levels are downregulated, allowing p73 to rise and to exert its proapoptotic effect. All these results implies that Itch is able to keep low p73 protein levels in unstressed conditions, similarly to Mdm2 pathway on p53.

Another important role in p73-dependent induction of cell death and in the regulation of p73 protein stability regulation is played by YAP. (Strano et al., 2001). The interaction between YAP and p73 results in p73 half-life prolongation and prevention of proteasomal degradation of p73, by competing with Itch to bind p73 (Levy et al., 2007). Interestingly, upon DNA-damage intracellular YAP protein levels are stabilized by c-Abl-dependent phosphorylation of YAP (Levy et al., 2008), therefore creating a circuit that mediates stabilization of p73 in a condition in which cell cycle arrest is desiderable. In contrast to the c-Abl function on YAP, the pro-survival Akt kinase, impairs YAP from associating with p73, leading to the attenuation of p73 transactivation and apoptotic activity (Basu et al., 2003; Strano et al., 2005).It has been found that ΔNp73 is selectively degraded in response to DNA damage, in a Itch-independent manner (Rossi et al., 2005; Maisse et al., 2004). Thus, it could be speculated that Itch is prevents apoptosis by keeping the levels of all p73 isoforms low under normal physiological conditions, and that this block is released for TAp73 upon DNA damage. Anyway, to date the mechanism by which TAp73 and ΔNp73 are differentially regulated remains an open question. To these ends, a possible role for c-Jun in differentially regulating the accumulation of these p73 isoforms in response to DNA damage as well as their steady state half-lives has been recently reported (Toh et al., 2004). Moreover, evidence for another mechanism deputed to p73 degradation, in addition to Itch, emerged from the observation that p73 degradation requires the integrity of the ubiquitin-like Nedd8 conjugation pathway (Bernassola et al., 2004).

2. Aim of the project

p73 is a structural and functional homologue of the tumor suppressor transcription factor p53, which binds to canonical p53 DNA-binding sites, activates transcription from p53-responsive promoters and, hence, induces cell cycle arrest and apoptosis (reviewed in Melino et al., 2002). In contrast to p53, p73 exists as several distinct protein isoforms (α-η) generated by alternative splicing at the C-terminal (De Laurenzi et al, 1998, 1999 and 2000). Additionally, the p73 gene has two distinct promoters; the first promoter (P1) yields proteins possessing an N-terminal transactivation domain (TAD), the transcriptionally active (TA) isoforms. The usage of the alternative internal promoter (P2) gives rise to N-terminally truncated proteins (ΔN isoforms), which lack the TAD and, as a result, act as dominant negative inhibitors of p53 and TAp73 tumor suppressive functions (Kaghad et al., 1997; Ueda et al., 1999). Thus, TA- and ΔNp73 isoforms display antagonistic functions: the TAp73 variants largely mimic p53 suppressive activities, while the ΔNp73 proteins promote cell survival and exhibit oncogenic properties (Yang et al., 2000a; Grob et al., 2001; Sayan et al., 2004). As a result of the opposite activities exerted by TA- and ΔNp73 proteins, the balance between cell death and survival, particularly in cells harboring p53 mutations, will crucially depend on the relative proportions of the two isoforms (Melino et al., 2002). Similarly to p53, p73 expression is maintained at low levels in mammalian cells, and its cellular induction and activation is mainly controlled at the post-translational level. p73 is polyubiquitylated *in vivo* and degraded via the proteasomal proteolytic system (Bernassola et al., 2004; Maisse et al., 2004). It has been previously reported that p73 ubiquitylation is catalysed by the HECT type E3 ubiquitin ligase (E3) Itch (Rossi et al., 2005). In unstressed cells, Itch targets both TA- and ΔNp73 for protein ubiquitylation, thereby keeping their expression levels low under normal conditions. Following DNA damage treatment, TAp73 protein levels accumulate, while ΔNp73 is rapidly degraded, in an Itch-independent manner. In several tumor cell lines, the induction of TAp73 in response to chemotherapeutic drugs is, at least partially,

accomplished through Itch downregulation (Rossi et al., 2005). Our findings imply that different E3 ligases can account for p73 degradation in different conditions. On the basis of these evidences, my PhD project has been focused on ubiquitin-dependent degradation of p73, on one hand testing the possible implication of another E3 ligase activity in the regulation of p73 protein level, and on the other analyzing the molecular mechanisms of Itch self-ubiquitylation and investigating its possible involvement in the regulation of Itch protein stability.

3. Results/1

3.1 Analysis of Itch self-ubiquitylation molecular mechanism and its possible involvement in Itch protein stability

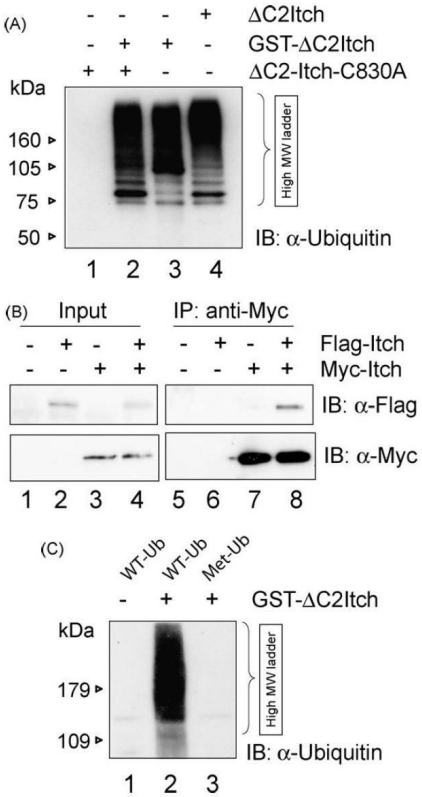
3.1.1 Itch self-polyubiquitylation occurs through an intermolecular mechanism

Consistent with previous reports (Rossi et al., 2005; Gao et al., 2004; Gallagher et al., 2006; Mouchantaf et al., 2006), Itch acts in concert with the E2 UbcH7 to promote its own ubiquitylation in a cell-free system (**Fig. 1A, lanes 2–4**). To further explore the molecular mechanism of Itch auto-modification, we utilized a mutant Itch in which the highly critical Cys residue in its HECT domain was mutated to Ala (Itch-C830A) (Rossi et al., 2005). The C830A mutation abrogates Itch ligase activity, and consequently, its auto-ubiquitylating capacity (Oberst et al., 2007). To assess whether Itch mediated auto-ubiquitylation occurs through an *in cis* or *in trans* reaction, we tested the ability of Itch-C830A to serve as a substrate for the wild-type enzyme in an *in vitro* ubiquitylation assay. Interestingly, mutated Itch was properly polyubiquitylated by the wild-type E3, demonstrating that Itch can catalyze the transfer of ubiquitin from its catalytic Cys to a second Itch molecule which serves as a substrate (**Fig. 1A, lane 2**). These results are in line with our previous observation that the catalytic inactive mutant is, at least moderately, ubiquitylated *in vivo*, likely due to the activity of endogenous Itch (Oberst et al., 2007).

3.1.2 Itch generates self-assembled polyubiquitin chains that require internal Lys residue 63 of ubiquitin

We next sought to determine the type of self-polyubiquitin chains generated by Itch. Utilizing an *in vitro* ubiquitylation assay,

Fig. 1 - Itch self-ubiquitylation acts through an in trans mechanism. ***(A)*** *The Itch-C830A catalytic inactive Itch mutant serves as a substrate for the ubiquitylation activity of wild-type Itch. The in vitro ubiquitylation reaction was performed using bacterially purified DC2-Itch-C830A (about 70 kDa) as a substrate for wild-type GST-DC2-Itch (about 100 kDa). The polyubiquitylation ladders of ΔC2Itch-C830 and GST-ΔC2Itch start at approximately 75 and 105 kDa, respectively, indicating that the self-ubiquitylation reaction involves two separate Itch molecules.* ***(B)*** *HEK293T cells were co-transfected using the calcium-phosphate method with flag-tagged- and myc-tagged-Itch. Forty-eight hour after transfection, cell lysates were immunoprecipitated with monoclonal anti-myc antibody and IB analysis was performed by using either anti-flag or anti-myc antibodies. Inputs and immunoprecipitates are shown on the left and right panel, respectively.* ***(C)*** *Bacterially purified GST-DC2Itch was subjected to an in vitro ubiquitylation reaction in the presence of either wild-type (lane 2) or Met-Ub (lane 3) that can only support mono-ubiquitylation. Ubiquitylated Itch was detected by IB using anti-ubiquitin antibody.*



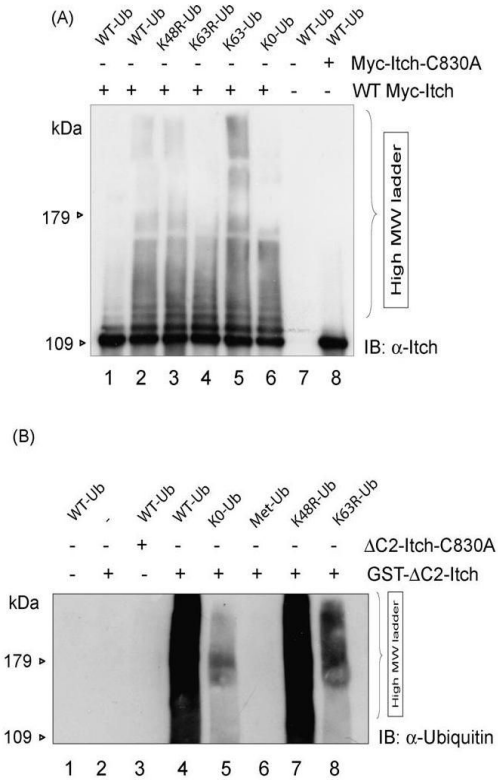


Fig. 2 – Itch self-polyubiquitylation involves Lys63 linkages. ***(A)*** *HEK293T cells were transfected with plasmids coding wildtype and C830A catalytic inactive Itch. After cell lysis, Itch was immunoprecipitated with anti-myc antibody, followed by an in vitro ubiquitylation assay. The ubiquitylation reaction was carried out using WT (lanes 1, 2, 7 and 8), K48R (lane 3), K63R (lane 4), K63 (lane 5), or K0 (lane 6) ubiquitin mutants for 90 min at 30 8C. As a negative control, the ubiquitylation reaction was stopped prior incubation at 30 8C (lane 1). Itch ubiquitylated forms were detected by IB using anti-Itch antibody.* ***(B)*** *Bacterially purified GST wild-type and C830A DC2Itch fusion proteins were subjected to an in vitro ubiquitylation assay in the presence of WT-Ub (lanes 1, 3, and 4), K0-Ub (lane 5), Met-Ub (lane 6), K48R-Ub (lane 7), or K63R-Ub (lane 8). The assay was analyzed by IB using anti-ubiquitin antibody.*

we found that immunopurified Itch is still able to catalyze its own ubiquitylation in the presence of a ubiquitin mutant lacking Lys48 (K48R-Ub) (**Fig. 2A, lane 3**). Similarly to a ubiquitin mutant lacking all Lys residues (K0-Ub), substitution of Lys63 with Arg (K63R-Ub) failed to support Itch autoubiquitylation (**Fig. 2A, compare lanes 4 and 6**). On the contrary, recombinant ubiquitin lacking all Lys residues but Lys63 (K63-Ub) was properly incorporated into self-polyubiquitin chains (**Fig. 2A, lane 5**). The relative increase in ubiquitin conjugates observed in the presence of K63-Ub (lane 5) could be explained by assuming that under *in vitro* conditions, Lys residues other than Lys63 could be partially utilized for polyubiquitin chain formation. These results indicate that Itch auto-ubiquitylation occurs through Lys63-linked polyubiquitin. To rule out the possibility that Lys63-linkages were catalyzed by a different E3 activity co-immunoprecipitating with Itch, we tested the ability of bacterially purified Itch to promote its own ubiquitylation in the presence of the a forementioned ubiquitin mutants. We indeed observed that the formation of polyubiquitin chains was abolished only by using the K63R-Ub mutant (**Fig. 2B, lane 8**), while Itch selfubiquitylation was retained in the presence of K48R-Ub (**Fig. 2B, lane 7**). All together, these findings demonstrate that Itch catalyzes the generation of self-ubiquitin chains that involve Lys63 of ubiquitin.

3.1.3 Itch is a stable protein

The inability of Itch to synthesize its own K48-based polyubiquitin chains, which is the signal for proteasome recognition, prompted us to analyze the half-life of endogenous Itch. We found that Itch is a highly stable polypeptide, whose decay rate is not appreciable up to 8 h of protein synthesis blockade (**Fig. 3A**). On the contrary, p21 protein levels declined 1 h after treatment (**Fig. 3A**). Furthermore, the steady state levels of endogenous Itch were not altered by either incubation with the proteasome inhibitor MG132, or following exposure to a panel of inhibitors of lysosomal hydrolases (**Fig. 3B**). Collectively, these findings indicate that endogenous Itch degradation is a relatively slow process. Under our conditions, Itch protein levels are not significantly regulated by either proteasome-

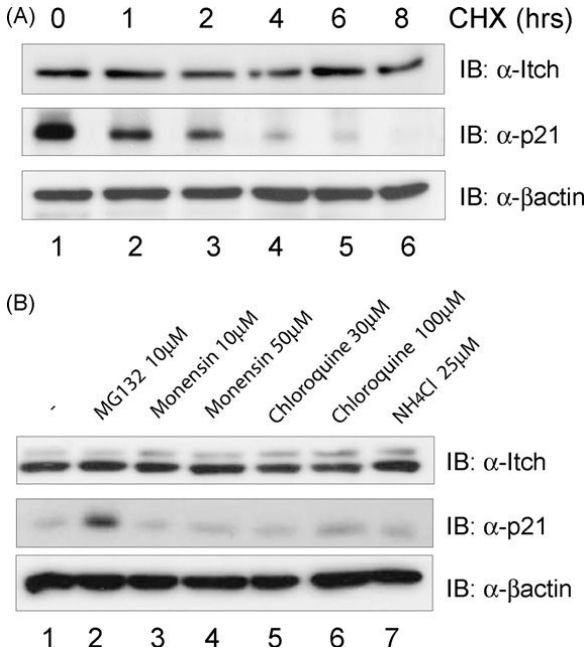


Fig. 3 – The steady state degradation of Itch is a relatively slow process. ***(A)*** *Determination of endogenous Itch protein half-life. HEK293T were treated with CHX (80 mg/ml) for the indicated time points. Cell lysates were analyzed by IB using anti-Itch, anti-p21 (as a positive control for protein synthesis blockade) and anti-b-actin antibodies.* ***(B)*** *Effect of proteasome and lysosomal inhibitors on the steady-state level of endogenous Itch. HEK293T cells were treated with proteasomal (MG132, lane 2) and lysosomal hydrolases (monensin, chloroquine and NH4Cl, lanes 3–7) inhibitors at the indicated concentrations for 6 h. Endogenous Itch steady-state levels were determined by IB with anti-Itch antibody. Protein levels of the cell cycle inhibitor p21 were assessed as a positive control for proteasomal degradation.*

dependent degradation or by lysosomal activities, and are unlikely governed by selfubiquitylation occurring *in vivo*.

3.1.4 Itch self-ubiquitylating activity of is not required for its degradation

To further rule out the involvement of Itch auto-ubiquitylating activity in the regulation of its degradation, we reconstituted Itch deficient MEFs with either wild-type or C830A mutant Itch, and their steady-state levels were monitored in the absence or in the presence of the proteasome inhibitor MG132. As shown in **Fig. 4A**, the two proteins display similar expression levels, and were equally insensitive to proteasome inhibition. As an internal control, we measured p21 and c-Jun induction in response to MG132 treatment. This result confirms that lack of enzymatic activity does not affect Itch protein stabilization. A comparison of their decay rate revealed that wild-type and C830A Itch are both highly stable polypeptides (**Fig. 4B**).

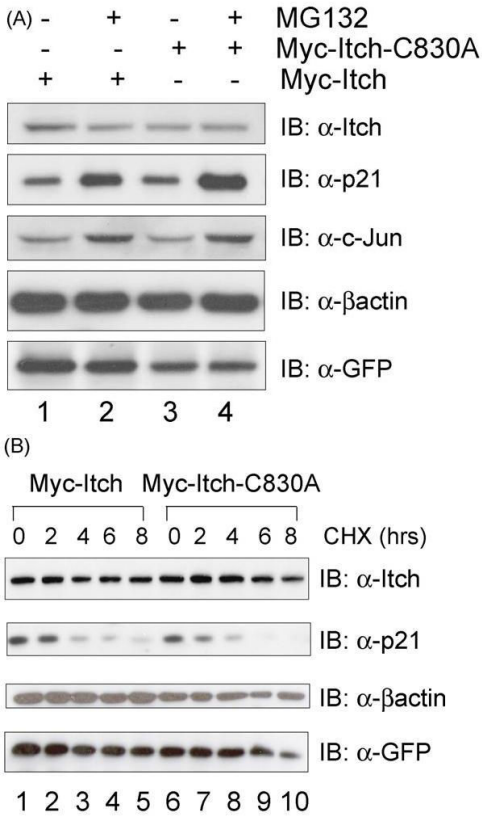


Fig. 4 – Lack of self-ubiquitylating activity does not affect Itch protein stability. ***(A)*** *Effect of the proteasome inhibition on the steady-state levels of wild-type and C830A catalytic inactive Itch. ItchS/S MEFs were co-transfected with GFP along with either wild-type or C830A catalytic inactive mutant myc-Itch. Twenty-four hours after transfection, cells were treated with 20 mM MG132 for 5 h, harvested and analyzed by IB by using anti-Itch antibody. GFP proteins levels were used to normalize transfection efficiency.* ***(B)*** *Protein decay rates of wild-type and C830A mutant Itch. ItchS/S MEFs were transfected as indicated in panel A. Twenty-four hours after transfection, cells were treated with 80 mg/ml CHX for the indicated times. Cell lysates have been examined by IB using anti-Itch antibody.*

3. Results/2

3.2 Testing the possible implication of Skip1-Cul1-FBXO45 (SCF^{FBXO45}) complex in the regulation of p73 protein levels

3.2.1 Cul1-dependent regulation of p73 protein level

In order to investigate the putative role of a Cullin-based E3 ligase in controlling p73 protein levels, we took advantage of a CHO-derived ts41 cell line harboring a temperature sensitive mutation in the APP-BP1 gene encoding the Nedd8 E1-activating enzyme (Handeli S & Weintraub H, 1992). Nedd8 is an ubiquitin-like protein, whose covalent attachment to cullins is required for the activity of Cullin-based E3 ligases (see above). We transfected HA-tagged p73 (HA-TAp73) into ts41 cells and analyzed by immunoblot (IB) p73 levels either at permissive (34 °C) or not permissive (40 °C) temperature. At 40 °C the neddylation pathway is inhibited, as shown by the lack of the nedd8 modification of Cul2 (**Fig. 1A**). In concomitance with the inactivation of the Nedd8 conjugation pathway, we observed an increase in p73 protein levels (**Fig. 1A**). This effect is specific for p73 since the other members of the p53 family, p63 and p53, are not regulated in the same manner (**Fig. 1B**). In humans there are five different cullin proteins, all of them requiring the Nedd8 modification to exert their function. We investigated which cullin proteins account for the increase of the p73 level observed in ts41 cells. We co-transfected HA-TAp73 with different dominant negative mutants of cullins and then analyzed p73 levels by IB. Expression of Cul1 dominant negative mutant, but not Cul2, Cul3, Cul4 and Cul5 dominant negative mutants, increased p73 levels (**Fig. 1C**) to an extent similar to that observed in the ts41 cell line at not permissive temperature, suggesting that a Cul1-associated activity is required for p73 protein stability.

Cul1 is the scaffold protein of the SCF complexes and many substrates of this class of E3s were found to be associated with Cul1(see above). Therefore, we tested whether a complex between TAp73 and Cul1 can be observed *in vivo*. We performed an *in vivo*

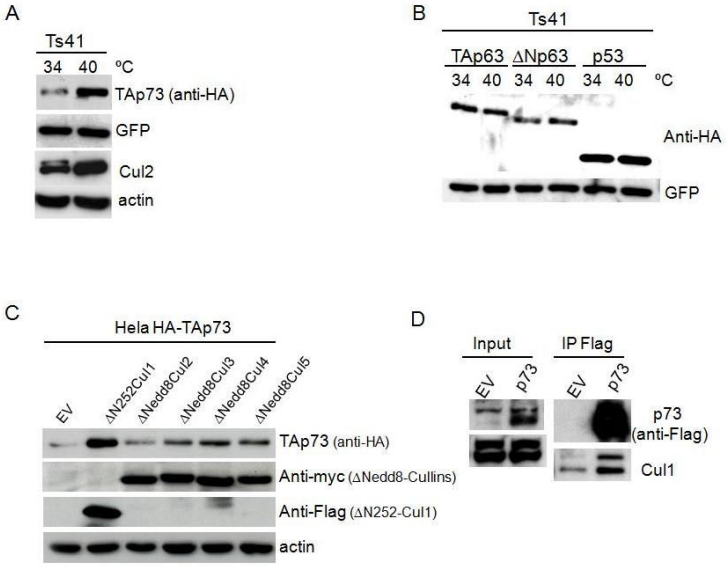


Fig. 1 - Cul1-associated activity regulates p73 protein levels.
(A) *ts41 cells were co-transfected at permissive temperature (34°C) with HA-tagged TAp73 (HA-TAp73) and GFP. Twenty-four hr post-transfection, cells were either left at the permissive temperature or shifted to non-permissive teperature (40°C) for 24 hrs. Cell lysates were immunoblotted with anti-HA and anti-actin antibodies. Blots were re-probed with antibodies against Cul-2 and GFP, as a control of impaired neddylation pathway and transfection efficiency, respectively.* ***(B)*** *ts41 cells were co-transfected with either HA-TAp63, HA-ΔNp63, HA-TAp63 or HA-p53 together with GFP. Cells were treated as in A, and cell lysates were analyzed by immunoblotting (IB) with antibodies against HA and GFP.* ***(C)*** *HeLa cells were transfected with a plasmid coding HA-TAp73 in the absence or in the presence of the Flag-tagged ΔN252Cul1 mutant, and different myc-tagged Nedd8 defective mutants of Cul2, Cul3, Cul4 and Cul5. Cellular extracts were analyzed by IB using anti-HA, anti-Flag and anti-myc antibodies. Blots were re-probed with anti-actin antibody as loading control.* ***(D)*** *HEK293T cells were transfected with Flag-tagged TAp73 and cell extracts were immunoprecipitated with anti-Flag antibody and analyzed with anti-Flag and anti-Cul1 antibodies.*

co-immunoprecipitation (co-IP) experiments and found that endogenous Cul1 indeed binds Flag-tagged TAp73 (**Fig. 1D**). All these results imply a role for an SCF complex in the regulation of p73 protein level.

3.2.2 The F-box protein FBXO45 binds specifically to p73

In *C. elegans* the F-box protein FSN-1 controls germline apoptosis by regulating the p53 ortholog, CEP-1(Gao et al., 2008). Among the 520 predicted F-box proteins of *C.elegans*, FSN1 is among the few to be conserved through evolution and its human ortholog is FBXO45 (Kipreos & Pagano, 2000). This has prompted us to investigate whether FBXO45 could interact with p73. To this aim, we screened different human F-box proteins for their ability to bind to p73. Flag-tagged versions of F-box proteins were co-transfected with HA-tagged p73 into HEK293T cells (adding the proteasome inhibitor MG132 for six hours before harvesting the cells) and then immunoprecipitated to evaluate their interaction with p73. We found that both wild type FBXO45 and its F-box deleted mutant (ΔF-FBXO45) interacted with p73 (**Fig. 2A**), while related F-box proteins such as FBXO11, FBX04 and FBX028 did not. Similarly, various additional F-box proteins (Skp2, bTrcp, FBXW7 and FBXO9) did not co-immunoprecipitate p73 either (**Fig. 2A**). Importantly, FBXO45 binds to both TAp73 and ΔNp73 isoforms, but not to p53 (**Fig. 2B**), indicating the specificity of the interaction between FBXO45 and p73.

3.2.3 FBXO45 is recruited into a SCF complex

At a structural level, human FBXO45 is characterized by an N terminal F-box domain (spanning from amminoacids 39 to 82) and a SPRY domain (spanning amminoacids 152 to 284) (Fig. 3A).Only a few is known about F-box protein FBXO45. Its gene has been identified in 2004 as one of six genes encoding F-box proteins

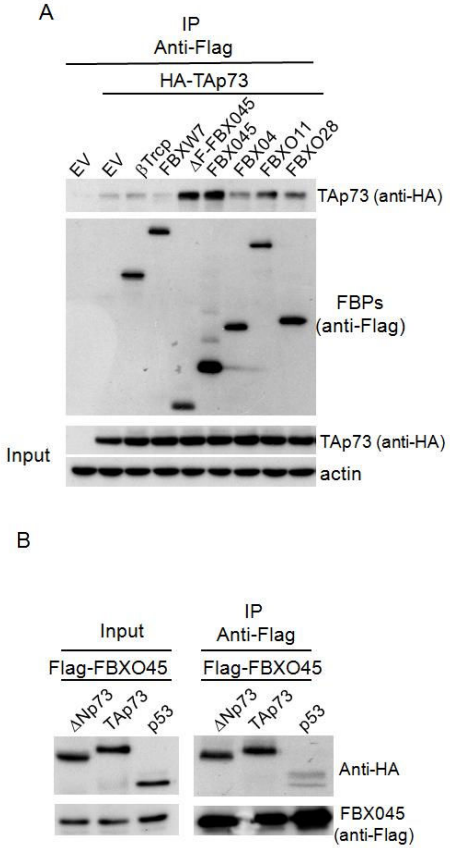


Fig. 2 - The F-box protein FBXO45 binds specifically to p73.
(A) *HEK293T cells were co-transfected with the indicated Flag tagged F-box protein constructs (FBPs) and HA-TAp73. During the last 6 hours before harvesting, cells were treated with 10 μM of the proteasome inhibitor MG132. Exogenous proteins were immunoprecipitated (IP) from cell extracts with anti-Flag resin and immunocomplexes were probed with antibodies to the indicated proteins. (EV= empty vector).* ***(B)*** *HEK293T cells were co-transfected with Flag-tagged FBXO45 (Flag-FBXO45) together with either HA-TAp73, HA-ΔNp73 or HA-p53. Cells extracts were IP using an Anti-Flag antibody and immunocomplexes were probed with antibodies to the indicated proteins.*

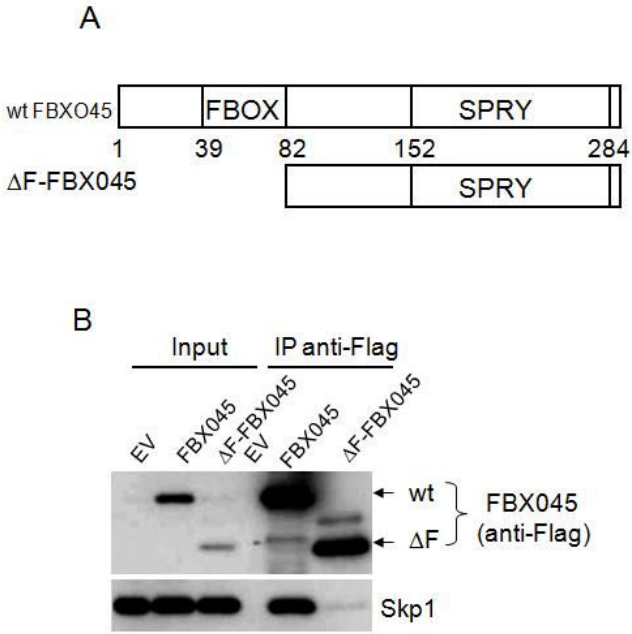


Fig. 3 – FBXO45 is recruited into a SCF complex. ***(A)*** *Structure of wild type FBXO45 and its F-box deleted mutant ΔF-FBXO45.* ***(B)*** *HEK293T cells were transfected with either wilde type Flag-FBXO45 or its mutant Flag-ΔF-FBXO45. Cell extracts were immunoprecipitated with an antibody anti-Flag and the immunocomplexes were analyzed by IB with antibodies to the indicated proteins*

appearing to be conserved in *C. elegans, D. melanogaster*, and mammals (Jin et al., 2004). Successively, it has been described as an estrogen-induced gene which codifies for a still orphan F-box protein (Yoshida, 2005). Its transcription start site is preceded by several estrogen receptors binding sequences, and it is composed by 3 exons; in fact, it has been shown that FBXO45 mRNA arises after stimulation with 17β-estradiol in MCF-7 cells (Yoshida, 2005). By using bioinformatics, FBXO45 is shown to be human ortholog of *C. elegans* FSN-1, with which it displays 54,9% amino acids identity (Jin et al., 2004; human FBXO45 accession number: NP_001099043; http://www.treefam.org/cgi-bin/TFseq.pl?id=ENSG00000174013; http://www.expasy.ch/cgi-bin/sim.pl?prot).

It is well established that the F-box domain accounts for the binding to the adaptor protein Skp1, while the other domains present in the F-box proteins are responsible for their interaction with substrates. To test whether FBXO45 is a component of the SCF complex *in vivo*, we transfected HEK293T cells with expression constructs for wild type Flag-tagged FBXO45 or ΔF-FBXO45. While wild type FBXO45 was able to interact with endogenous Skp1, the ΔF-FBXO45 mutant did not, indicating that the F-box domain of FBXO45 is functionally important to form an SCF complex *in vivo* (**Fig. 3B**).

3.2.4 FBXO45 induces the proteasome-dependent degradation of p73

Since the SCF complexes generally regulate the proteasome-dependent degradation of target proteins, we studied the effect of overexpressing FBXO45 on p73 levels. HA-TAp73 was co-transfected into neuroblasoma SH-SY5Y cells in the presence of either wild type Flag-tagged FBXO45 or ΔF-FBXO45. As shown in **Fig. 4A**, p73 protein levels were efficiently down regulated by the co-expression of wild type FBXO45, both under unstressed conditions and after DNA damage. Importantly, the ΔF-FBXO45 mutant, which is able to bind p73 (see **Fig. 2A**), was not capable to negatively modulate p73 levels, confirming the requirement of an

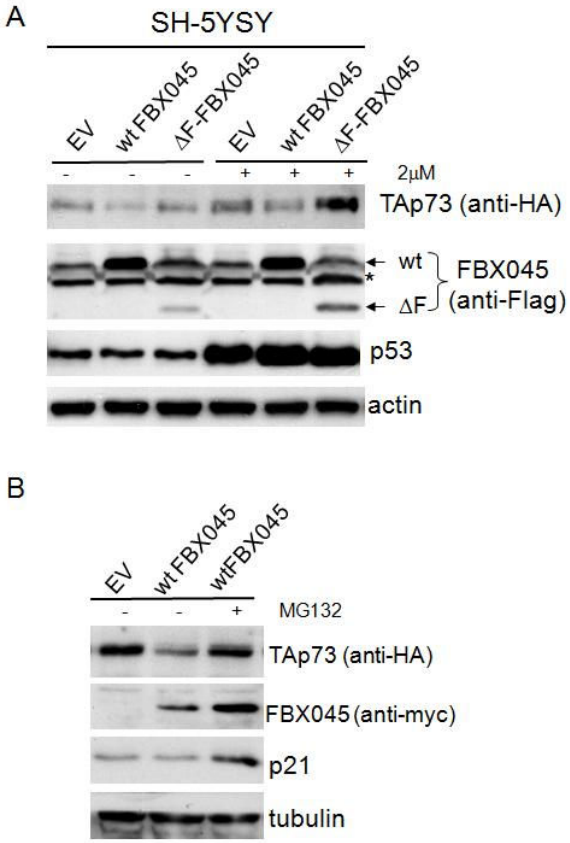


Fig. 4 - FBXO45 promotes the proteasome-dependent degradation of p73 ***(A)*** *SH-5YSY neuroblastoma cells were co transfected with HA-TAp73 together with either wild type Flag-FBXO45 or its mutant Flag-ΔF-FBXO45. Twenty-four hrs post-transfection, cells were either left untreated or treated with doxorubicin (2 μM) for 16 hrs. Cellular extracts were analyzed by IB using anti-HA and anti-Flag antibodies. Blots were re-probed with anti-actin as loading control. (EV= empty vector). Asteriks (*) represents a non-specific band.* ***(B)*** *The experiment was performed as in C, except that 10μM of MG132 was added where indicated.*

intact SCF^{FBXO45} complex for regulating p73 protein levels. To assess whether the FBXO45-mediated dowregulation of p73 is due to proteasome-dependent degradation, we treated SH-SY5Y cells expressing both wild type FBXO45 and p73 with the proteasome inhibitor MG132. As shown in **Fig. 4B**, exposure to MG132 totally rescued FBXO45-dependent downregulation of p73, demonstrating that FBXO45 controls the proteasome-dependent degradation of p73 *in vivo*.

3.2.5 SCF^{FBXO45} ubiquitylates p73 *in vivo* and *in vitro*

Because a major function of F-box proteins involves the ubiquitylation of their target substrates, we sought to determine whether FBXO45 ubiquitylates p73 in mammals cells. To this aim, we transfected the constructs expressing myc-tagged FBXO45, Flag-tagged p73 and HA-tagged Ubiquitin into SH-SY5Y cells. We then performed IP with anti-FLAG antibody-conjugated M2 beads under denaturing conditions to eliminate any p73-associated proteins through non-covalent bonds. Ubiquitin-conjugated p73 species were detected by IB with an anti-Flag antibody. As shown in **Fig. 5A**, the expression of FBXO45 enhanced the ubiquitylation of p73 as compared to the control vector (compare lane 2 to lane 4). We also reconstituted the ubiquitylation of p73 *in vitro* by using a semi-purified system. Flag-tagged wild type FBXO45 or its mutant ΔF-FBXO45 were transfected into HEK293T cells and then purified by IP with anti-Flag antibody. After extensive washes, the beads containing the SCF^{FBXO45} or $SCF^{\Delta F\text{-}FBXO45}$ complexes were mixed to an *in vitro* ubiquitylation reaction containing immunopurified TAp73. FBXO45, but not its F-box deleted mutant ΔF-FBXO45, is able to promote the ubiquitylation of purified p73 (**Fig. 5B**). A different F-box protein, FBXO11, was unable to trigger the ubiquitylation of p73, confirming the specificity of FBXO45. All these results indicate that FBXO45 promotes p73 ubiquitylation both *in vivo* and *in vitro* in an SCF-dependent manner.

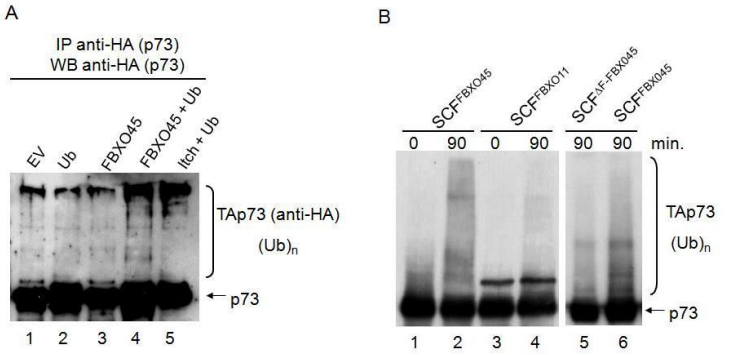


Fig. 5. SCF^{FBXO45} stimulates the ubiquitylation of p73 both *in vivo* and *in vitro*. ***(A)*** *In vivo ubiquitin ligation assay of TAp73 was conducted as described in materials and methods section. Briefly, SH-5YSY neuroblastoma cells were co-transfected with HA-TAp73 alone or in the presence of the following proteins: empty vector (EV, lane 1), myc-tagged ubiquitin (lane 2), Flag-FBXO45 (lane 3), Flag-FBXO45 and myc-ubiquitin (lane 4), Flag-Itch and myc-ubiquitin (lane 5). After 24 hours, cells were treated with 10μM of proteasome inhibitor MG132 for 6 hours and then collected. Cell extracts were subjected to immunoprecipitation prior denaturation of protein samples. The immunoicomplexes were resolved in SDS/PAGE and analyzed by IB using the antibodies for the indicated proteins.* ***(B)*** *In vitro ubiquitin ligation assay of immunopurified HA-TAp73 was conducted in the presence or absence of the following proteins: immunopurified SCF^{FBXO45}, immunopurified SCF^{FBXO11}, immunopurified SCF$^{\Delta F\text{-}FBXO45}$. Samples were incubated at 300C for 90 minutes except that in lanes 1 and 3 that were added to sample buffer. The bracket on the right side of the panel marks a ladder of bands corresponding to polyubiquitylated p73.*

3.2.6 Silencing of FBXO45 induces p73 stabilization and cell death in the absence of functional p53.

p73 activity controls different processes, including the apoptotic response to DNA damage and neuronal differentiation. In order to test whether the DNA damage response regulates FBXO45 expression, we performed an RT-PCR analysis in cells treated with doxorubicin, a DNA damage-inducing agent. **Fig. 6A** showed that FBXO45 expression was down regulated after DNA damage both in SH-5YSY cells and BT-20 breast cancer cells. On the contrary, we did not observe any significant variation of FBXO45 mRNA levels in SH-5YSY cells treated with retinoic acid to induce neuronal differentiation (data not shown). The results presented above prompted us to test whether the depletion of FBXO45 could increase p73 protein levels and potentiate thus the apoptotic response to a DNA damage agent. To this end, we employed the small interfering RNA (siRNA) technique to reduce the expression of FBXO45 in the BT-20 cell line, which harbors a mutation in the p53 locus, thus, avoiding p53-dependent apoptosis. We first tested two different siRNA oligos for their ability to downregulate FBXO45. Both oligos were able to decrease FBXO45 expression at the mRNA level and induced a concomitante accumulation of p73 (**Fig. 6B**). Importantly, FBXO45 depletion does not affect the mRNA level of p73 (**Fig. 6B**), indicating that it specifically controls p73 protein stability.

To determine whether FBXO45 downregulation could sensitize cells to apoptosis, we treated the FBXO45-depleted cells with doxorubicin and we measured the apoptotic index after DNA damage. In concomitance with the upregulation of p73, FBXO45-depleted cells showed a marked increase in the apoptotic index (**Fig. 6C**), indicating thus that FBXO45 could regulate cell death in the absence of functional p53.

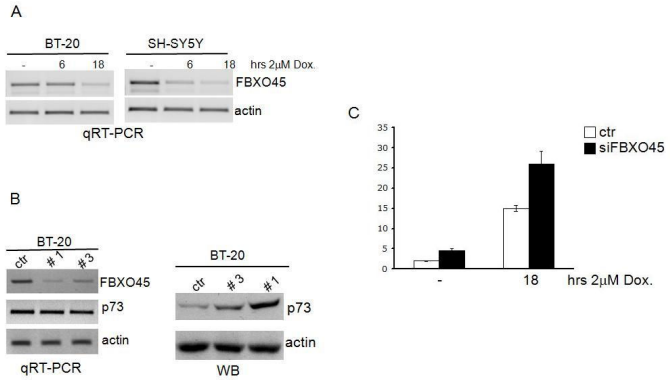


Fig. 6. FBXO45 expression is down regulated after DNA damage and its depletion by siRNA sensitizes BT-20 breast cancer cell line to doxorubicin-induced cell death. (*A*), *BT-20 breast cancer cell line and SH-5YSY neuroblastoma cells were either left untreated or treated with doxorubicin (2 μM) for 6 and 18 hrs. Total RNA was extracted from the cells and qRT-PCR was performed utilizing specific primer for FBXO45 and actin.* ***(B)*** *BT-20 cells were transfected twice with siRNA molecules to a non-relevant mRNA (ctr) or to two different oligos specific for FBXO45 (#1 and #3). Forty- hours after transfection, total RNA was isolated and qRT-PCR was performed as described in A. Concomitantly cell extracts were subjected to IB using the indicated antibody.* ***(C)*** *BT-20 cells transfected with siRNA as in B, were either left untreated or treated with doxorubicin (2 μM) for 18 hrs. The percentage of sub-G1 cells was measured by FACS analysis. A representative results is shown (mean ± s.d. n=3)*

4. Experimental procedures

4.1 Cell culture, transfection conditions, and reagents

Human embryonic kidney HEK293T, Itch-/- mouse embryonic fibroblasts (MEFs), human epithelial carcinoma HeLa, human neuroblastoma SH-5YSY and human breast cancer cell line BT-20 were maintained at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). Chinese hamster ovary (CHO) Ts41 cell line was maintained at 34°C, and then shifted to non-permissive temperature (40°C) to inhibits the neddylation pathway. Transfections were performed using the calcium phosphate method for HEK293T, Effectene (Qiagen) for HeLa, BT-20 and MEFs and Lipofectamine LTX (Invitrogen) for SH-5YSY, according to the manufacturer's instructions. Cells have been treated for 6 hours with 10 □M proteasome inhibitor MG132 (Calbiochem) and/or with 2 □M Doxorubicin hydrochloride (Sigma) when appropriate. The following pcDNA3.1 expression constructs were kindly provided by Vincenzo De Laurenzi (University of Rome "Tor Vergata") for all p53, p63 and p73 constructs; Mario Rossi (Medical Research Council, University of Leicester) for Itch (myc-Itch wt and myc-ItchC830A); Michele Pagano (NYU School of Medicine) for F-box proteins (myc-FBXO45, Flag-βTrCP, Flag-FBXO4, Flag-FBXO28, Flag-Skp2). The pcDNA3.1 expression vector for wild type Flag-tagged FBXO45 has been obtained by subcloning the FBXO45 cDNA into the pcDNA3.1 Flag B vector. The ΔF-FBXO45 deletion mutant has been generated by PCR utilizing the following primers: 5'-CGGGATCCATGGATTACAAGGAGGATGACGACGATAAGTGCGCCCGCAGCCTGGCAGAAGAG-3' and 5'-CCGCTCGAGTCATCCGTCCAAAGGTTTTCCAAGGTAAACC-3'.

4.2 Reagents and antibodies

The following reagents were used: cycloheximide (CHX, Sigma), monensin (Sigma), NH4Cl (Sigma), chloroquine (Sigma), MG132 (Calbiochem), N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES, Sigma), Adenosine 50-triphosphate (Roche), ubiquitin aldehyde (Sigma). The E1 ubiquitin activating enzyme (E1), the E2 UbcH7, and recombinant purified wild-type (WT) and mutant ubiquitins were from Boston Biochem. The ubiquitin mutants were either molecules in which all Lys residues were changed to Arg (K0-Ub), or in which all Lys residues but one were replaced by Arg (K63-Ub), or in which individual Lys residues were mutated to Arg (K48R-Ub, K63RUb).The following antibodies were used: mouse monoclonal anti-Itch (BD Biosciences), mouse monoclonal anti-Myc (Cell Signaling Technology Inc.), mouse monoclonal anti-p21 (Sigma), mousemonoclonal anti-actin (Sigma), rabbit polyclonal anti-Flag (Sigma), mouse monoclonal anti-ubiquitin (Zymed), mouse monoclonal anti-HA (ascites, Covance), rabbit polyclonal anti-GFP (Roche), rabbit polyclonal anti-Cul2 (Zymed), mouse monoclonal anti-b-actin (Sigma), mouse monoclonal anti-Myc (Cell Signaling), rabbit polyclonal anti-Flag (Sigma), mouse monoclonal anti-Cul1 (Zymed), mouse monoclonal anti-p53 (DO-1, Santa Cruz), mouse monoclonal anti-p73 (Imgenex) and rabbit polyclonal anti-p73 (described in Rossi et al., 2005). For Itch expression in bacterial cells, we employed a modified construct (GST-C2-Itch) lacking the N-terminal region to avoid solubility problems due to the presence of the C2 domain. C2-Itch retains direct substrate interaction abilities as well as a functional catalytic domain. On the contrary, all plasmids utilized for the expression of wild-type and C830A-Itch in eukaryotic cells encode the full-length proteins.

4.3 Measurement of Itch half-life

Itch turnover rate was determined using Cycloheximide (CHX) inhibition of protein synthesis. Itch-/- MEFs were transiently transfected with either wild-type or C830A mutant Itch. Twenty-four hours after transfection CHX (Sigma) was added to the culture

media to a final concentration of 80 g/ml. Cells were harvested at indicated time points, and equal amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblot (IB). The same protocol was used to analyze the half-life of endogenous Itch in HEK293T cells.

4.4 Immunoblot analysis, immunoprecipitation and antibodies

Immunoblot analysis has been performed using whole cell extracts obtained by lysing the cell pellet with Triton Buffer (50 mM Tris-Hcl pH 7.5, 250 mM NaCl, 50 mM NaF, 1mM EDTA 1 pH 8, 0,1% Triton), supplemented with proteases and phosphatases inhibitors, and with 20 M N-ethylmaleimide (NEM, Sigma) when required. Proteins were subsequently separated by SDS/PAGE, blotted onto PVDF membranes (Millipore) and then blocked with PBS-T (Phosphate-buffered saline and 0,1%Tween-20) containing 5% non-fat dry milk for 1 hr at room temperature (RT). The incubation with primary antibodies was performed for 2 hrs at RT, followed by appropriate horseradish peroxidase-conjugated secondary antibody for 1 hr at RT. After extensive washing, detection was performed with the ECL Western Blot Chemiluminescence Reagent (Perkin Elmer).

Immunoprecipitation has been performed by preclearing 1 mg of whole cells lysates with protein A/G-Sepharose beads (GE Healthcare) for 3 hrs and then by incubating over-night at 4°C with the appropriate amount of antibody per sample. Finally, the immunocomplexes have been absorbed on protein A/G-Sepharose beads, washed six times in Triton lysis buffer and used for in vitro ubiquitylation assay or eluted by boiling in SDS loading buffer.

4.5 Ubiquitylation assays

In vivo ubiquitylation assay was performed using SH-5YSY neuroblastoma cells, transiently transfected with cDNAs encoding human Flag-tagged p73 (Flag-p73), myc-tagged FBXO45

(myc-FBXO45) and HA-tagged ubiquitin (HA-Ub) and treated with MG132 before collecting. Cells were lysed in denaturing condition (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) in the presence of 20 □M N-ethylmaleimide (NEM, Sigma), and Flag-p73 has been immunoprecipited as described above. Polyubiquitylated p73 species were detected using anti-Flag antibody.

In vitro ubiquitylation assay was carried out using SCF^{FBXO45} complexes immunopurified from HEK393T cells transfected with His-tagged Skip and Flag-tagged FBXO45 using the method described above. The beads containing the SCF^{FBXO45} immunocomplexes were washed twice with equilibration buffer (25 mM Tris-Hcl pH 7,5, 50 mM NaCl, 1 mM EDTA, 0,01% Triton and 10% glycerol) and then added to the following reaction: 30 ng of E1, 0.5 μg of UbcH3, 0.5 μg of UbcH5, 5 mg of ubiquitin in the ubiquitylation buffer (2,5 mM Tris·HCl, 0,7 mM DTT, 4 mM ATP, 10 mM MgCl2, 0.1 mM ubiquitin aldheyde). The HA-tagged p73 (HA-p73) protein used as substrate for the reaction has been purified by immunoprecipitation with the HA antibody and then eluted with the HA peptide (1 mg/ml). The assays have been carried out for 90 minutes at 30°C, then the immunocomplexes were eluted by boiling in SDS loading buffer and loaded in SDS/PAGE.

The in vitro Itch self-ubiquitylation assay was carried out as previously described (Rossi et al., 2005) using bacterially purified proteins (gently provided by Mario Rossi, Medical Research Council, University of Leicester). Briefly, the ubiquitylation reaction mixture contained 25mM Tris–HCl (pH 8.0), 100 mM NaCl,1 mM DTT, 2.5 mM ATP, 4mM MgCl2, 2 l of bacterial purified wheat E1, 0.1 g of a human UbcH7, 100 ng of bacterially purified recombinant wild-type or C830A mutant GST-C2-Itch,and 5 g of Flag-tagged ubiquitin. After incubation for 90 min at30 8C, the reactions were terminated by adding SDS loading buffer, resolved by SDS-PAGE, followed by IB with antiubiquitin or anti-Itch antibodies. Alternatively, the in vitro ubiquitylation reaction was performed utilizing Flag-Itch immunopurified from HEK393T cells after transient transfection. Briefly, Itch immunocomplexeswere washed twice with equilibration buffer (25 mMTris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.01% Triton and10% glycerol). For each sample, 30 ng of E1, 0.5 mg of UbcH7and 5 g of ubiquitin were added to the ubiquitylation reaction mixture (2.5 mM Tris–HCl, 0.7

mM DTT, 4 mMATP, 10 mM MgCl2, 0.1 mM ubiquitin aldehyde). After incubation at 30 8C for the indicated time points, selfubiquitylated Itch was eluted from the beads by boiling in SDS loading buffer, resolved by SDS/PAGE, followed by IB anti-Itch.

4.6 siRNA-mediated silencing of FBXO45 and detection of apoptosis

SH-5YSY and BT-20 cells were seeded at a density of 1.4 x 105 cells/well in a 6-well plate and transfected with oligos twice (at 24 and 48 hours after plating) using Oligofectamine (Invitrogen) according to manufacturer's instructions. Two different 21-nucleotide RNA oligos were used, targeting the follow FBXO45 cDNA sequences: 5'-CAGACGTTACTATTATCCCTA-3' and 5'-CTGGTGGACAATAATCTACTA-3'. A 21-nt siRNA duplex corresponding to a non-relevant gene (scramble) was used as control. Cells were collected after 48 hours and lysates were subjected to Western Blot. To evaluate the efficiency of silencing, we measured the FBXO45 mRNA level by RT-PCR using total RNA extracted by FBXO45-depleted cells. Alternatively, after siRNA transfection, we transfected the siRNA expressing cells with vector encoding Flag tagged FBXO45. After 24 hours, cells were lysed and total cell extracts were analyzed by IB using the Flag antibody.

Quantification of sub-G1 population was performed by FACS analysis of propidium iodide-staining nuclei (as described in Nicoletti et al., 1991), carried out in a FACScan flow cytometer (Becton Dickinson) using the CELLQuest software system.

5. Discussion

In recent years, ubiquitylation has attracted increasing attention in the context of the regulation of apoptosis. Ubiquitylation functions primarily to label proteins for degradation by the protease 26S proteasome through the covalent binding of ubiquitin chains, and is highly specific (Pickart, 2001). Briefly, a cascade of reactions that is catalyzed by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzimes (E2) and ubiquitin ligases (E3) is required to attach the small ubiquitin moiety to a lysine residue of an acceptor protein. Ubiquitylation is a reversible process, in spite of proteasomal degradation of ubiquitylated proteins is irreversible, and therefore ideally suited for controlling unidirectional pathways such as cell death. Important regulators of apoptosis, including the Bcl-2 family of proteins, the IAPs, and regulators of the inhibitor of nuclear factor-kB kinase (IKK) have been identified as new substrates of the ubiquitylation system, in addition to other already known substrates of the proteasome, such as p53.

p73 is a structural and functional homologue of the tumor suppressor transcription factor p53, which binds to canonical p53 DNA-binding sites, activates transcription from p53-responsive promoters and, hence, induces cell cycle arrest and apoptosis. p73 also controls other biological processes, including tumorigenesis and neuronal differentiation (reviewed in Melino et al., 2002). In contrast to p53, p73 exists as several distinct protein isoforms (α-η) generated by alternative splicing at the C-terminal (De Laurenzi et al, 1998, 1999 and 2000). Additionally, the p73 gene has two distinct promoters; the first promoter (P1) yields proteins possessing an N-terminal transactivation domain (TAD), the transcriptionally active (TA) isoforms. Instead, the usage of the alternative internal promoter (P2) gives rise to N-terminally truncated proteins (ΔN isoforms), which lack the TAD and thus act as dominant negative inhibitors of p53 and TAp73 tumor suppressive functions (Kaghad et al., 1997; Ueda et al., 1999). Thus, TA- and ΔNp73 isoforms display antagonistic functions. As a result of the opposite activities exerted by TA- and ΔNp73 proteins, the balance between cell death and survival, particularly in cells harboring p53 mutations, will crucially

depend on the relative proportions of the two isoforms (Melino et al., 2002).

Similarly to p53, p73 expression is maintained at low levels in mammalian cells, and its cellular induction and activation is mainly controlled at the post-translational level. Knowledge on the mechanisms regulating p73 levels in basal conditions as well as in response to stress is essential to design new therapies that require p73 induction. p73 is polyubiquitylated *in vivo* and degraded via the proteasomal proteolytic system (Bernassola et al., 2004; Maisse et al., 2004). Our group has previously demonstrated that the HECT E3 ubiquitin ligase Itch is capable to polyubiquitylate p73 and induce its degradation in a proteasome-dependent manner. (Rossi et al., 2005). In unstressed cells, Itch targets both TA- and ΔNp73 for protein ubiquitylation, thereby keeping their expression levels low under normal conditions. Following DNA damage treatment, TAp73 protein levels accumulate, while ΔNp73 is rapidly degraded, in an Itch-independent manner. In several tumor cell lines, the induction of TAp73 in response to chemotherapeutic drugs is, at least partially, accomplished through Itch downregulation (Rossi et al., 2005). Thus, our findings implied that different E3 ligases can account for p73 degradation in different conditions. Furthermore, it has been previously reported that Itch is capable to undergo self-ubiquitylation, although its physiological role has not been clearly elucidated (Gao et al., 2004; Mouchantaf et al., 2006). Self-ubiquitylation activity of E3 ligases (E3s) has been previously described for both RING-type and HECT-type E3s (Bruce et al., 2008). It is thought to mainly act as a regulatory mechanism that controls the abundance of E3s by marking them for degradation (Yang et al, 2000b; Fang et al, 2000). On the basis of these evidences, my PhD project has been focused on ubiquitin-dependent degradation of p73, on the one hand testing the possible implication of another E3 ligase activity in the regulation of p73 protein level, and on the other hand analyzing the molecular mechanisms of Itch self-ubiquitylation and investigating its possible involvement in the regulation of Itch protein stability.

Though Itch auto-ubiquitylation has been previously reported, we have further characterized the molecular mechanisms underlying this post-translational modification. In the present study, we provide evidences that Itch engages an intermolecular reaction generating Lys63 polyubiquitin chains, and that this auto-

modification does not regulate Itch protein stability. Consistently with the non proteolytic self-ubiquitylating activity of Itch, the catalytic inactive C830A mutant was as stable as the wild-type counterpart, and displayed similar sensitivity to the proteasome inhibitor treatment. All together, these findings definitely prove that, in contrast to other E3s (Yang et al., 2000; Fang et al., 2000), the self-polyubiquitin chains generated by Itch do not serve as either proteasome or lysosome targeting.

The apparent discrepancy of our conclusions with the recently reported stabilizing activity manifested by the deubiquitylating enzyme USP9X on Itch protein levels (Mouchantaf et al., 2000), may be explained by assuming that Itch may serve as a substrate for other not yet discovered E3s, and that the ubiquitin protease would utilize alternative non-self-catalyzed ubiquitin chains as a substrate. Moreover, the analysis of the primary structure of Itch reveals that different lysine residues are spread along the entire protein, and the inability of Itch to catalyze discrete self-monoubiquitylated bands in the presence of the K0-Ub mutant fits with the hypothesis that these different Lys residues of Itch can be utilized as acceptor sites for ubiquitin transfer. Without the knowledge of the specific Lys residues acting as polyubiquitin chains anchors, there is an open question remaining about the possible role(s) of Itch self-modification. Though HECT-type E3s have been considered to be constitutively active and regulated only at the level of target binding, there is emerging evidence showing that they are finely controlled either by phosphorylation (Gao et al., 2004; Yang et al., 2006) or through the association with adaptor proteins (Oberst et a., 2007; McGill et al., 2003). We speculate that auto-ubiquitylation could serve as a further regulatory mechanism, which may affect Itch function at different levels. One possibility would be direct interference with its catalytic activity. Alternatively, self-ubiquitylation could induce a conformational change influencing the posttranslational modification pattern of Itch. For instance, upon ubiquitylation, Itch phosphorylation state could be modified, hence allowing an alteration of the inhibitory intramolecular interactions regulating its catalytic activation (Gallagher et al., 2006). As a result of both molecular mechanisms, Itch capacity to conjugate ubiquitin to protein targets would be affected. A similar outcome for self-ubiquitylation has been described for another E3 ligase, reporting that the ability of the

RING-finger E3 Ring1B to monoubiquitylate histone H2A is increased upon self-ubiquitylation of Ring1B, which generates atypical Lys6, Lys27 and Lys48 mixed ubiquitin chains (Ben-Saadon et al., 2006). In addition, it has been described that Lys63-linked auto-ubiquitylation of TRAF6 is required for its ability to ubiquitylate NEMO, and for subsequent IKK and NF-B activation (Lamothe et al., 2007). Both mono- and poly-ubiquitylation of protein substrates have been associated with internalization, sorting and changes in their subcellular localization (Geetha et al., 2005a and 2005b; Trotman et al., 2007). Hence, ubiquitin conjugation may represent a signal for Itch to translocate to distinct cellular compartments, which ultimately, would modify its accessibility to certain substrate molecules. Itch is predominantly localized to early and late endosomal compartments and lysosomes, though a small fraction displays a perinuclear and nuclear distribution (Marchese et al., 2003; Muchantaf et al, 2006). Numerous Itch substrates are transcription factors mainly residing in the nuclear compartment. Hence, self-ubiquitylation may represent an auto-regulatory mechanism controlling Itch cytoplasmic-nuclear shuffling. Efforts are underway to examine the possible contribution of Itch auto-catalytic ubiquitylation to these processes. A significant number of Itch molecular targets are important regulators of apoptotic cell death (Gao et al., 2004; Rossi et al., 2005; Chang et al., 2006). As a result, their inappropriate removal, due to altered Itch regulation would be likely linked to cellular transformation. It is therefore crucial to unveil the physiological relevance of Itch self-ubiquitylation, and the data shown in this thesis leads to a further investigation.

The evidences previously discussed, that suggest the existence of other E3 ligases besides Itch, open the way to the second part of this PhD thesis work, in which we made efforts to verify whereas another E3 ligase is implicated in p73 degradation and which one it could be. We tested the hypothesis that a member of cullin-based subfamily of RING E3 ubiquitin ligases could be responsible for the ubiquitylation of p73. The cullins proteins, a subunit of cullin-based E3s, need to be neddylated in order to have active complexes; coherently with this we found that NEDD8 system is required for p73 degradation, suggesting that p73 ubiquitinylation might be catalyzed by a cullin-based E3 ligase.

Moreover, we found that the expression of the dominant negative mutant of Cul1, but not of Cul2, Cul3, Cul4 and Cul5, induces p73 protein stabilization, suggesting that a Cul1-associated activity is required for p73 protein stability. Furthermore, we shown that Cul1 co-immunoprecipitates with TAp73. Given that Cul1 is a subunit of SCF complexes subfamily, all these results imply a role for an SCF complex in the regulation of p73 protein level.

The substrate specificity key subunit of SCF complexes is constituted by F-box proteins. They recruit specific substrates to the E3 ligase SCF complex, thus targeting them to proteasome-dependent degradation (Cardozo & Pagano, 2004). Despite the large number of F-box proteins, only nine human SCF ubiquitin ligases have well-established substrates, many of which are involved in cell cycle control, apoptosis and DNA damage response (Peschiaroli et al., 2006; Frescas & Pagano, 2008). A recent report has described that the worm ortholog of FBXO45, the F-box protein FSN-1, is implicated in germline apoptosis through the regulation of the apoptotic activity of CEP-1 (Gao et al., 2008). Like its vertebrate relatives, the *C. elegans* p53-like gene CEP-1 is the key regulator of the cell death after genotoxic stress (Schumacher et al., 2001; Derry et al., 2001). CEP-1 expression is induced after DNA damage and its activation controls germline apoptosis. Gao and co-authors reported that CEP-1 activity is strictly regulated by the SCF complex SCF^{FSN-1}. FSN-1 null mutants exhibit higher induction of CEP-1 levels after genotoxic stress and concomitantly a higher apoptotic index (Gao et al., 2008). On the basis of these evidences, we try to bring out whereas FBX45 could be involved in SCF-dependent degradation of p73. Our data indicates that exogenously expressed FBXO45 is able to bind to both TA and ΔN isoforms with similar affinities, suggesting that FBXO45, like Itch, is not able to discriminate between these two isoforms. In agreement with this, FBXO45 overexpression promotes the proteasome-dependent degradation of both TA and ΔN isoforms. Structurally the DNA binding domain of CEP-1 is similar to the one of human p53, but its C-terminus contains a SAM domain that resembles the C-terminus of vertebrate p63 and p73 (Ou et al., 2007), suggesting its similarity with the p63/p73 ancestor. Although we cannot completely rule out a possible involvement of FBXO45 in the regulation of p63 and p53 activity, our data indicate that FBXO45 controls specifically the

protein degradation of p73. In fact, FBXO45 is able to interact with p73, but not with p53. Moreover, we found that the protein level of p53 and p63 are not affected by the inhibition of the neddylation pathway, suggesting that the activity of the SCF complex is not implicated in the regulation of p53/p63 stability. Accordingly, it has previously described that the neddylation pathway regulates the transcriptional activity of p53 but not its stability (Abida et al., 2007).

As mentioned before, in *C.elegans* the genetic depletion of FSN-1 or Cul1 induces germline apoptosis through the increase of CEP-1 protein level. In line with these data, we showed that the siRNA-mediated depletion of FBXO45 increases p73 protein levels and sensitizes cells to DNA damage inducing apoptosis. From all these data, it appears that an SCF-dependent regulation of the p53 family members is a mechanism that has been conserved though evolution, reinforcing the important physiological role played by this pathway.

The F-box proteins generally recruit substrates in a phosphorylation-dependent manner (Skowyra et al., 1997) . Different kinases have been reported to be able to phosphorylate p73. In response to DNA-damaging agents, the non-receptor tyrosine kinase c-Abl phosphorylates p73 at Tyr99 leading to an increase in p73 stability (Gong et al., 1999). Moreover, p73 was found to interact with cyclin dependent kinases such as cyclin A/cdk2 and cyclin E/cdk2 and threonine 86 of p73 could be phosphorylated by these kinases *in vitro* (Gaiddon et al., 2003). More recently, the cell cycle regulated kinase Plk1 has been shown to interact with p73 and to induce its phosphorylation at Threonine 27 (Koida et al., 2008; Soond et al., 2008). Importantly, the Plk1-dependent phosphorylation of p73 seems to negatively regulate p73 protein stability, even though a formal demonstration of this issue is still missing (Soond et al., 2008). It will be important in the future to determine whether the FBXO45-dependent degradation of p73 is dependent on some of the above-mentioned phosphorylation events or on another kinase activity.

In conclusion, we demonstrated that the SCF^{FBXO45} complex binds to, ubiquitylates and stimulates the proteasome-dependent degradation of p73. Since FBXO45 depletion sensitizes cell to apoptosis in a p53 null background, we elucidate a potential new mean that could be used to develop new strategies aimed to

potentiate the apoptotic response of cancer cells following chemotherapy.

It is known that degradation of p53 family members is regulated through distinct molecular mechanisms; for example, the E3 ligase MDM2 has a critical function in the negative regulation of p53 activity in vivo (Montes de Oca Luna, et al., 1995), but it does not affect to p63 and p73 protein levels (Bálint et al., 1999). This is surprising given the high degree of structural and functional homology among p53 family members and may suggest that the evolutionary divergence of degradation mechanisms has evolved to ensure that the possible deregulation of this process would not concomitantly affect the entire p53 family.

6. Conclusions

p73, a member of the p53 family, is a transcription factor controlling different biological processes, including cell death, tumorigenesis and neuronal differentiation. Knowledge on the mechanisms regulating p73 levels in basal conditions as well as in response to stress is essential to design new therapies that require p73 induction. Our group has previously demonstrated that the HECT E3 ubiquitin ligase Itch is capable to polyubiquitylate p73 and induce its degradation in a proteasome-dependent manner. The work performed in this PhD project has been focused on ubiquitin-dependent degradation of p73, analyzing the molecular mechanisms of Itch self-ubiquitylation and its possible role in Itch protein stability, and testing the possible implication of an E3 ubiquitin ligase, different from Itch, in the regulation of p73 protein level.

In this study, we provide evidences that Itch engages an intermolecular reaction generating Lys63 polyubiquitin chains, and that this auto-modification does not regulate Itch protein stability. Furthermore, our findings demonstrate that the self-polyubiquitin chains generated by Itch do not serve as either proteasome or lysosome targeting. Both mono- and poly-ubiquitylation of protein substrates have been associated with internalization, sorting and changes in their subcellular localization. Hence, ubiquitin conjugation might represent a signal for Itch to translocate to distinct cellular compartments, which ultimately, would modify its accessibility to certain substrate molecules. Itch is predominantly localized to early and late endosomal compartments and lysosomes. Numerous Itch substrates are transcription factors mainly residing in the nuclear compartment. Hence, self-ubiquitylation may represent an auto-regulatory mechanism controlling Itch cytoplasmic-nuclear shuffling.

The F-box proteins recruit specific substrates to the E3 ligase SCF complex, thus targeting them to proteasome-dependent degradation. Despite the large number of F-box proteins, only nine human SCF ubiquitin ligases have well-established substrates. Here we show that the F-box protein FBXO45 is recruited into SCF^{Fbx45} complex and is able to bind to p73 promoting its ubiquitylation and its proteasome-

dependent degradation. Since FBXO45 depletion sensitizes cell to apoptosis, we elucidate a new, conserved mechanism that could be potentially used to develop new strategies aimed to potentiate the apoptotic response of cancer cells following chemotherapy.

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Summary

Background and aim

p73 is a structural and functional homologue of the tumor suppressor transcription factor p53, which binds to canonical p53 DNA-binding sites, activates transcription from p53-responsive promoters and, hence, induces cell cycle arrest and apoptosis (reviewed in Melino et al., 2002). In contrast to p53, p73 exists as several distinct protein isoforms (α-η) generated by alternative splicing at the C-terminal (De Laurenzi et al, 1998, 1999 and 2000). Additionally, the p73 gene has two distinct promoters; the first promoter (P1) yields proteins possessing an N-terminal transactivation domain (TAD), the transcriptionally active (TA) isoforms. The usage of the alternative internal promoter (P2) gives rise to N-terminally truncated proteins (ΔN isoforms), which lack the TAD and, as a result, act as dominant negative inhibitors of p53 and TAp73 tumor suppressive functions (Kaghad et al., 1997; Ueda et al., 1999). Thus, TA- and ΔNp73 isoforms display antagonistic functions: the TAp73 variants largely mimic p53 suppressive activities, while the ΔNp73 proteins promote cell survival and exhibit oncogenic properties (Yang et al., 2000a; Grob et al., 2001; Sayan et al., 2004). As a result of the opposite activities exerted by TA- and ΔNp73 proteins, the balance between cell death and survival, particularly in cells harboring p53 mutations, will crucially depend on the relative proportions of the two isoforms (Melino et al., 2002). Similarly to p53, p73 expression is maintained at low levels in mammalian cells, and its cellular induction and activation is mainly controlled at the post-translational level. p73 is polyubiquitylated *in vivo* and degraded via the proteasomal proteolytic system (Bernassola et al., 2004; Maisse et al., 2004). It has been previously reported that p73 ubiquitylation is catalysed by the HECT type E3 ubiquitin ligase (E3) Itch (Rossi et al., 2005). In unstressed cells, Itch targets both TA- and ΔNp73 for protein ubiquitylation, thereby keeping their expression levels low under normal conditions. Following DNA damage treatment, TAp73 protein levels accumulate, while ΔNp73 is rapidly degraded, in an Itch-independent manner. In several tumor

cell lines, the induction of TAp73 in response to chemotherapeutic drugs is, at least partially, accomplished through Itch downregulation (Rossi et al., 2005). Our findings imply that different E3 ligases can account for p73 degradation in different conditions. On the basis of these evidences, my PhD project has been focused on ubiquitin-dependent degradation of p73, by one side testing the possible implication of another E3 ligase activity in the regulation of p73 protein level, and by the other analyzing the molecular mechanisms of Itch self-ubiquitylation and investigating its possible involvement in the regulation of Itch protein stability.

Results

Self-ubiquitylation activity of E3 ligases (E3s) has been previously described for both RING-type and HECT-type E3s (Bruce et al., 2008). It is thought to mainly act as a regulatory mechanism that controls the abundance of E3s by marking them for degradation (Yang et al, 2000b; Fang et al, 2000). It has been previously reported that Itch is capable to undergo self-ubiquitylation although its physiological role has not been clearly elucidated (Gao et al., 2004; Mouchantaf et al., 2006). In the context of the first part of my project, we tested whether Itch self-ubiquitination could affect its protein stability. We demonstrated that Itch generates self-assembled Lysine-63 linked polyubiquitin chains, a signal generally not involved in targeting proteins for proteasome-dependent degradation. Consistently with this, we shown that Itch is a high stable protein, whose levels are not significantly affected by treatment by either proteasome or lysosome inhibitors. Furthermore, we demonstrated that the decay rate of a catalytic inactive Itch mutant, which is devoided of self-ubiquitylating activity, is indistinguishable from the one of the wild-type protein. All these results demonstrate that Itch self-ubiquitylation activity does not regulate its protein stability. As discussed above, the evidence that Itch is responsible for keeping both TAp73 and ΔNp73 levels low under normal condition but not in response to DNA damage suggests the involvement of another pathway to target p73 for degradation (Rossi et al., 2005). Additionally, it has been described that in C. elegans, the regulation of the p53-like protein CEP-1 is controlled by the F-box protein named FSN-1 (Gao et al., 2008).

Among 520 genome-predicted F-box proteins, FSN1 is one of the few to be conserved through evolution, and its human ortholog is FBXO45. F-box proteins represent the substrate targeting subunit of a class of RING-type E3 ubiquitin ligases known as Skp1-Cul1-F-box complexes (SCF). Taken together, these data led us to test the involvement of SCF^{FBXO45} complex in the regulation of p73 stability. We firstly proved the existence of a functional relation between SCF complex and p73 by demonstrating that the expression of the dominant negative of Cul1, but not the other cullins, stabilizes p73. Subsequentially, we found that SCF^{FBXO45} specifically interacts with p73, both TAp73 and ΔNp73 isoforms, suggesting that, similarly to Itch, FBXO45 is not able to discriminate between these two. Given that the major function of F-box proteins involves the ubiquitination of their target proteins, we sought to determine whether FBXO45 ubiquitinates p73 in mammals cells. We demonstrated indeed that FBXO45 significantly stimulates the ubiquitination of p73, both *in vitro* and *in vivo*. Significantly, on one hand the overexpression of FBXO5 induces the proteasome-dependent degradation of p73, and on the other the silencing of FBXO45 through RNA interference results in accumulation of p73. Moreover we found that FBXO45, similarly to Itch, is down-regulated in response to DNA damage, allowing thus p73 levels to increase in response to stress.

Conclusions

p73, a member of the p53 family, is a transcription factor controlling different biological processes, including cell death, tumorigenesis and neuronal differentiation. Knowledge on the mechanisms regulating p73 levels in basal conditions as well as in response to stress is essential to design new therapies that require p73 induction. Our group has previously demonstrated that the HECT E3 ubiquitin ligase Itch is capable to polyubiquitylate p73 and induce its degradation in a proteasome-dependent manner. The work performed in this PhD project has been focused on ubiquitin-dependent degradation of p73, analyzing the molecular mechanisms of Itch self-ubiquitylation and its possible role in Itch protein stability, and testing the possible implication of an E3 ubiquitin ligase, different from Itch, in the regulation of p73 protein level.

In this study, we provide evidences that Itch engages an intermolecular reaction generating Lys63 polyubiquitin chains, and

that this auto-modification does not regulate Itch protein stability. Furthermore, our findings demonstrate that the self-polyubiquitin chains generated by Itch do not serve as either proteasome or lysosome targeting. Both mono- and poly-ubiquitylation of protein substrates have been associated with internalization, sorting and changes in their subcellular localization. Hence, ubiquitin conjugation might represent a signal for Itch to translocate to distinct cellular compartments, which ultimately, would modify its accessibility to certain substrate molecules. Itch is predominantly localized to early and late endosomal compartments and lysosomes. Numerous Itch substrates are transcription factors mainly residing in the nuclear compartment. Hence, self-ubiquitylation may represent an auto-regulatory mechanism controlling Itch cytoplasmic-nuclear shuffling.

The F-box proteins recruit specific substrates to the E3 ligase SCF complex, thus targeting them to proteasome-dependent degradation. Despite the large number of F-box proteins, only nine human SCF ubiquitin ligases have well-established substrates. Here we show that the F-box protein FBXO45 is recruited into SCF^{Fbx45} complex and is able to bind to p73 promoting its ubiquitylation and its proteasome-dependent degradation. Since FBXO45 depletion sensitizes cell to apoptosis, we elucidate a new, conserved mechanism that could be potentially used to develop new strategies aimed to potentiate the apoptotic response of cancer cells following chemotherapy.

Riassunto

Presentazione del tema di ricerca e scopo del lavoro

p73 è un omologo strutturale e funzionale del soppressore tumorale p53, in grado di legare siti canonici di binding riconosciuti da p53, attivare la trascrizione attraverso promotori responsivi a p53, e quindi indurre arresto del ciclo cellulare e apoptosi (funzioni riassunte in Melino et al., 2002). Al contrario di p53, p73 possiede diverse isoforme (α-η) generate attraverso splicing alternativo all'espremità C-terminale (De Laurenzi et al, 1998, 1999 and 2000). Inoltre, il gene *TP73* presenta due distinti promotori, il primo dei quail (P1) genera delle isoforme che contengono all'N-terminale il dominio di transattivazione (TAD), e che sono dunque in grado di indurre transattivazione (isoforme TA). L'utilizzo di un promotore alternativo interno (P2) produce isoforme tronche all'estremità N-terminale (isoforme ΔN), che sono prive del TAD e dunque agiscono come inibitori dominanti negativi delle funzioni di soppressori tumorali ascrivibili a p53 e a p73 (Kaghad et al., 1997; Ueda et al., 1999). Dunque, le isoforme TAp73 e ΔNp73 mostrano funzioni opposte: le isoforme TAp73 mimano le attività di p53, mentre le isoforme ΔN promuovono la sopravvivenza cellulare e mostrano proprietà oncogeniche (Yang et al., 2000a; Grob et al., 2001; Sayan et al., 2004). Ne risulta che l'equilibrio tra sopravvivenza e morte cellulare, in particolare in cellule che possiedono mutazioni per p53, dipende dal rapporto tra le due isoforme (Melino et al., 2002). Come p53, l'espressione di p73 è manenuta a livelli bassi nelle cellule di mammifero, e la sua induzione e attivazione è prevalentemente controllata a livello post-traduzionale. p73 è poliubiquitinata *in vivo* e degradata attraverso il proteasoma (Bernassola et al., 2004; Maisse et al., 2004). È stato riportato che l'ubiquitinazione di p73 è catalizzata da Itch, una E3 ubiquitina ligasi (E3) di tipo HECT (Rossi et al., 2005). In condizioni normali. Itch ubiquitina sia TAp73 che ΔNp73, mantenendo dunque bassa la loro abbondanza nelle cellule. A seguito di stress genotossico, i livelli proteici di TAp73 aumentano, mentre ΔNp73 è rapidamente degradato, in maniera Itch-indipendente. In diverse linee tumorali, l'induzione di TAp73 in risposta a chemioterapici è ottenuta almeno parzialmente attraverso

la downregolazione di Itch (Rossi et al., 2005). I nostri precedenti risultati suggeriscono dunque che diverse E3 ligasi possano essere responsabili della degradazione ubiquitina-dipendente di p73 in differenti condizioni. Sulla base di ciò, il mio progetto di dottorato ha riguardato la degradazione ubiquitina-dipendente di p73, da una parte testando l'ipotesi dell'esistenza di di un'altra E3 ligasi la cui attività sia implicata nella regolazione dei livelli di p73, e dall'altra analizzando i meccanismi molecolari alla base dell'autoubiquitinazione di Itch e il suo possibile coinvolgimento nella regolazione della sua stabilità proteica.

Risultati

L'autoubiquitinazione di E3 ligasi è stata precedentemente descritta sia per ligasi di tipo RING che di tipo HECT (Bruce et al., 2008). Si ritiene che agisca prevalentemente come un meccanismo regolatorio che controlla l'abbondanza delle E3 marcandole per la degradazione attraverso il proteasoma (Yang et al, 2000b; Fang et al, 2000). È stato descritto che Itch ha la capacità di catalizzare la sua autoubiquitinazione, sebbene il suo ruolo fisiologico non sia stato chiarito (Gao et al., 2004; Mouchantaf et al., 2006). Nella prima parte del mio progetto di dottorato, ci siamo chiesti se l'autoubiquitinazione di Itch potesse influire sulla sua stabilità proteica. Abbiamo dimostrato che Itch è in grado di generare su un'altra molecola di Itch catene di poliubiquitina costruite attraverso la Lisina 63 presente nell'ubiquitina. Catene di poliubiquitina così assemblate costituiscono un segnale che non è coinvolto nella marcatura delle proteine per la degradazione proteasoma-dipendente. Coerentemente con ciò, abbiamo dimostrato che Itch è una proteina molto stabile, i cui livelli sono sono modificati in maniera significativa da trattamento con inibitori del proteasoma o del lisosoma. Inoltre, abbiamo dimostrato che il tasso di decadimento del mutante di Itch cataliticamente inattivo (ovvero incapace di autoubiquitinazione), è indistinguibile da quello della proteina wild type. Tutti questi risultati dimostrano che l'autoubiquitinazione di Itch non regola la stabilità proteica di questa E3 ubiquitina ligasi. Come già discusso, il fatto che in condizioni normali Itch sia responsabile del mantenimento a bassi livelli sia di TAp73 che di ΔNp73, ma non in seguito a danno al DNA, suggerisce il coinvolgimento di almeno un altro pathway responsabile della

degradazione di p73 (Rossi et al., 2005). Inoltre, è stato descritto che, in *C. elegans*, la regolazione dell'ortologo di p53, CEP-1, è controllata da una proteina F-box denominata FSN1 (Gao et al., 2008). Tra circa 520 F-box protein predette nel genoma di *C. elegans*, FSN-1 è una delle poche conservate attraverso l'evoluzione, e il suo ortologo umano è FBXO45. Le proteine F-box rappresentano la subunità responsabile del riconoscimento specifico del substrato di una sottofamiglia di E3 di tipo RING nota come complesso Skp1-Cul1-F-box (SCF). Tutti insieme questi dati ci hanno spinto a indagare il possibile coinvolgimento del complesso SCF^{FBXO45} nella regolazione della stabilità di p73. In primo luogo abbiamo verificato l'esistenza di una relazione funzionale tra il complesso SCF e p73 dimostrando che l'espressione del mutante dominante negativo di uno dei componendi dell'SCF, la cullina Cul1, stabilizza p73, al contrario delle altre culline. Successivamente, abbiamo dimostrato che SCF^{FBXO45} interagisce in maniera specifica sia con TAp73 che con ΔNp73, suggerendo che, in maniera simile ad Itch, FBXO45 non è in grado di distinguere tra le due isoforme. Poiché la funzione principale delle proteine F-box riguarda l'ubiquitinazione dei loro substrati, abbiamo verificato se FBXO45 ubiquitinasse p73 nelle cellule di mammifero. Abbiamo dimostrato che FBX045 stimola significativamente l'ubiquitinazione di p73, sia *in vitro* che *in vivo*. Inoltre, abbiamo osservato che FBXO45, come Itch, è downregolata in risposta a danno al DNA, permettendo che i livelli di p73 aumentino.

Conclusioni

p73, un membro della famiglia di p53, è un fattore di trascrizione che controlla diversi processi biologici, tra cui la morte cellulare, la tumorigenesi e il differenziamento neuronale. Conoscere i meccanismi che regolano i livelli di p73 in condizioni basali e in risposta allo stress cellulare è essenziale per progettare nuove terapie che richiedano l'induzione di p73. Il nostro gruppo ha precedentemente dimostrato che l'E3 ubiquitina ligasi di tipo HECT denominata Itch è in grado di poliubiquitinare p73 ed indurre la sua degradazione attraverso il proteasoma. Il lavoro riguardante questo progetto di dottorato è stato incentrato da una parte sulla degradazione ubiquitina-dipendente di p73, analizzando i meccanismi molecolari dell'autoubiquitinazione di Itch e il suo

possibile ruolo nella stabilità proteica di Itch stesso, e dall'altra testando il possibile coinvolgimento di una E3 ubiquitina ligasi diversa da Itch nella regolazione dei livelli proteici di p73.
In questo lavoro, abbiamo dimostrato che Itch è impegnata in una reazione intermolecolare che genera catene di poliubiquitina sintetizzate impiegando la Lys63 dell'ubiquitina stessa, e che questa auto-modificazione non regola la stabilità proteica di Itch. Inoltre, i nostri dati dimostrano che l'auto-poliubiquitinazione di Itch non conduce Itch stessa alla degradazione proteasoma- o lisosoma-dipendente.
Sia la mono che la poliubiquitinazione delle proteine è stata associata con internalizzazione, smistamento e con cambiamenti nella localizzazione subcellulare. Per cui l'ubiquitinazione di Itch potrebbe rappresentare un segnale per la sua traslocazione in altri compartimenti cellulari, e dunque modificare il suo accesso ad alcuni suoi substrati. Itch è infatti localizzata in maniera predominante negli endosomi precoci e tardivi e nei lisosomi. Numerosi substrati di Itch sono fattori di trascrizione che si trovano dunque prevalentemente nel compartimento nucleare. Quindi, l'autoubiquitinazione potrebbe rappresentare un meccanismo autoregolatorio che controlla il trasporto di Itch tra il citoplasma e il nucleo.
Le proteine F-box reclutano substrati specifici al complesso E3 ligasi Skp1-Cul1-F-box (SCF) ed quindi alla degradazione proteasoma-dipendente. Malgrado il gran numero di proteine F-box esistenti, solo di nove di esse si conoscono approfonditamente i substrati. In questa tesi abbiamo dimostrato che la proteina F-box FBXO45, è parte di un complesso SCF ed è capace di legare p73, di cui promuove l'ubiquitilazione e la degradazione proteasoma-dipendente. Poiché il silenziamento di FBXO45 sensibilizza le cellule all'apoptosi, noi abbiamo descritto un nuovo meccanismo, conservato attraverso l'evoluzione, che può essere potenzialmente oggetto dello sviluppo di nuove strategie per aumentare la risposta apoptotica in cellule cancerose attraverso la chemioterapia.

8. Index