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Functional analysis of Skp2 regarding p21 regulation in the developing nervous system of the mouse: *Skp2* elimination promotes Mdm2/Mdm4-mediated proteasomal degradation of p21.

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Introduction

1 - The cell cycle

The cell cycle is a standardized sequence of events, by which one cell becomes two cells. In general, a cell cycle needs a starter stimulus and then proceeds through defined steps, called cell cycle phases. The cell cycle usually comprises 4 phases: G1, S, G2 and M. After G1 a cell can proceed in a state of replicative quiescence called G0 phase. In each of these phases there are main positive regulators, called cyclins, which trigger the activation of cyclin dependent kinases (Cdks). The main negative regulators, called cyclin-dependent kinase inhibitors (CKIs), in turn bind to the cyclin-Cdk complexes or to the free Cdks, inhibiting or restraining their enzymatic activity. The outcome of this balance determines whether the cell progresses into the next cell cycle phase, or if it stops transiently or permanently [Alberts et al., 2002].

1.1 - Positive regulators of the cell cycle and Cdks

In G1 phase (gap 1) the so called “Restriction point” [reviewed in Planas-Silva and Weinberg, 1997] is a point of no return. Before the restriction point, the cell cycle can be activated by growth factors, while after that point the cell is committed to cycle. The crucial positive factor responsible for cell cycle progression is cyclin D, the cyclin that couples to Cdk4 and Cdk6 in G1 phase. When these Cdks become active, they can phosphorylate a group of pocket proteins, among which are the Retinoblastoma protein (pRb), p130 and p107. In their resting, unphosphorylated form, these are associated with proteins of the E2F family and sequester them. E2F proteins are transcription factors, responsible for the transcription of cyclin E: this cyclin binds Cdk2, giving rise to an activation cascade that prepares the cell for S phase. One important regulator of cell cycle progression is c-Myc; it belongs to the Myc family of transcription factors. Members of this family can promote

transcription of cyclinD, cyclinE and Cdc25 phosphatase, which is able to remove inhibitory phosporylations from Cdk2. Once the CyclinE-Cdk2 complex reaches the activation threshold, a positive feedback begins; this gives rise to the S phase (synthesis), where the cell duplicates its genetic information. While DNA is replicated, histones are also transcribed and translated, to bind the newly synthesized copy of the genome [reviewed in Bartek and Lukas, 2001].

Following S phase, during G2 phase (gap 2) the cell prepares the cell to carry out mitosis, by protein synthesis and reorganization of both duplicated DNA and enlarged cellular structures. The timing of this transition is given by the nuclear translocation of CyclinB1-Cdk1 complexes; these proteins reside in the cytoplasm during G2 phase, but they become active only when imported in the nucleus [reviewed in Lindqvist et al., 2009], where this Cyclin-Cdk complex triggers M phase [reviewed in O'Farrell, 2001].

During M phase (mitosis) the cell reorganizes most of its structures to divide into two daughter cells: the nuclear envelope is dissolved, while chromatin is condensed into chromosomes. These are packaged and bound to the cytoskeleton, which also reorganizes. Through a series of fixed steps the whole cellular structure is split into two daughter cells [reviewed in Thompson et al, 2010]. Usually, a cell that began a cycle can only end up in carrying out a successful mitosis or dying by apoptosis or mitotic catastrophe [Zhou and Elledge, 2000]. However, under peculiar conditions a cell is able to undergo endoreplication [reviewed in Edgar and Orr-Weaver, 2001]. Endoreplication consists of finishing a cell cycle without completing M phase: cytokinesis is omitted and the cell retains double amounts of genetic material and cellular structures.

1.2 - Negative regulators of the cell cycle and CKIs

Cell cycle proression can be negatively regulated in response to different stimuli; these trigger signaling cascades that lead to activation of Cdk inhibitors (CKIs) that can act during particular cell cycle phases.

Cdks are inhibited by two classes of CKIs [reviewed in Sherr and Roberts, 1999]. One group is the INK4 family, including $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, which specifically target Cdk4 and Cdk6 and therefore act during G1 phase. Another group is the CIP/KIP family, including $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which inhibit a broad spectrum of Cdks. Within this last family of inhibitors, p21 [reviewed in Gartel et al., 1996] is predominantly associated with DNA damage response and senescence. p57 [reviewed in Pateras et al., 2009] possesses many functions that are not yet completely understood. p27 has a prominent role in preventing cell cycle progression during G1 phase. Indeed, p27 can inhibit all the G1 Cdks [reviewed in Koff and Polyak, 1995] and is responsible for keeping cells in G1 phase. Moreover p27 has been shown to be responsible for a permanent exit from the cell cycle, leading to G0 phase [Ladha et al., 1998].

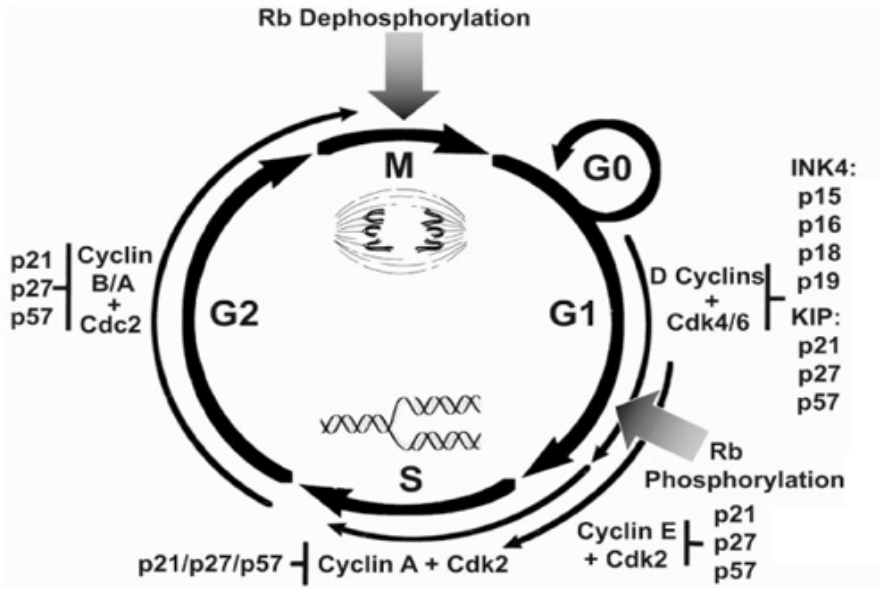


Figure 1 [from Donovan and Slingerland, 2000]. Scheme of the cell cycle, showing the principal cyclin-Cdk complexes and CKIs relative to each phase.

2 - Cell cycle checkpoints and DNA damage

While these CKIs regulate cell cycle exit physiologically, cell cycle arrest can also be activated due to checkpoint activation after insults. DNA damage is the predominant and most important activator of checkpoints. Depending on the kind of damage, different pathways can be activated: one fundamental distinction is between the formation of structural abnormalities or breaks in one DNA strand, or the breakage of both DNA strands [reviewed in Harrison and Haber, 2006]. The second kind of damage is more critical, as it involves a complete disruption in DNA strands cohesion. The DNA strands can be broken in consequence to the exposure to chemical or physical agents, and the most efficient source of double strand breaks (DSBs) is ionizing irradiation. DSBs cause an intense damage response, based on the Ataxia-Telangiectasia-mutated (ATM) protein pathway, which involves the activation of the downstream Checkpoint kinase 2 [Rotman and Shiloh, 1999]. ATM in turn activates many other transducers, amongst which is fundamental the tumor suppressor protein p53, a component of all DSB response checkpoints [reviewed in Giono and Manfredi, 2006]. p53 activation can either lead to cell cycle stop via p21, or to apoptosis [reviewed in Cuddihy and Bristow, 2004].

2.1 - p53

The p53 gene product is the main responsible protein that keeps genomic and functional integrity in every cell, since it can trigger apoptosis as a consequence to irrecoverable damage or aberrations. p53 is often mutated in tumors and cell lines and, upon p53 inactivation or loss, cells become tolerant regarding cellular survival, thus gaining the ability to grow increasingly different from a healthy normal cell [Donehower et al., 1992].

After DNA damage, p53 coordinates DNA repair with cell cycle progression [Friedman et al., 1993]. The p53 protein contains three domains (figure 2): an N-terminal acidic transactivating domain [Fields and Jang, 1990.], a central DNA-binding domain and a complex C-terminal domain [Anderson et al., 1997], that harbors nuclear localization sequences and a homotetramerization domain.

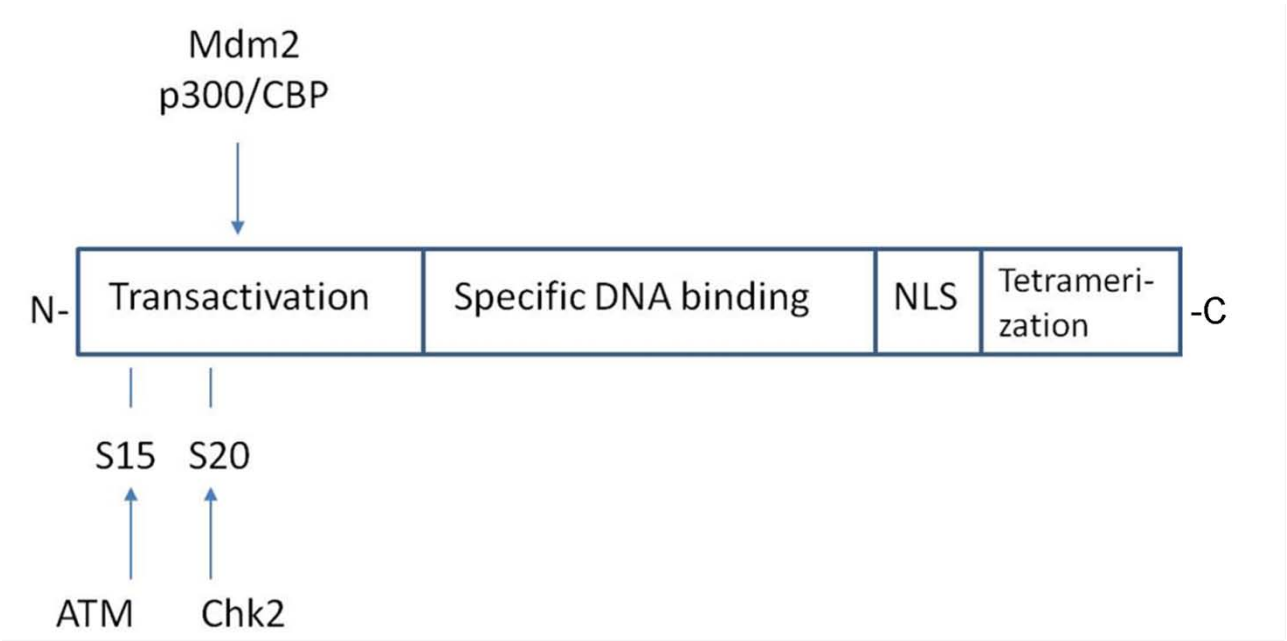


Figure 2. Structure of the p53 protein, showing protein domains, DNA damage related phosphorylation sites and the principal regulatory interactions by p300 and Mdm2.

p53 is subject to a fine regulation, which involves post translational modifications that alter its stabilization, binding capacity, and subcellular localization.

In response to DNA damage, especially DSBs, ATM is autophosphorylated and its kinase activity is fully activated [Bakkenist and Kastan, 2003]. As a consequence, ATM is capable of phosphorylating p53 both directly and indirectly: direct phosphorylation of p53 by ATM occurs on serine 15 (in human) [Canman et al., 1998]. ATM also activates the protein kinase Chk2 and, subsequently, activated Chk2 phosphorylates p53 on serine 20 [Shieh et al., 2000]. These phosphorylation events inhibit the destruction of p53, leading to an accumulation of protein and an increased p53 half-life of 1-2 hours, in contrast to a half-

life of 10-20 minutes in the absence of DNA damage [reviewed in Lakin and Jackson, 1999]. Nuclear export of stabilized p53 is also inhibited following DNA damage, allowing p53 to accumulate in the nucleus [Jimenez et al., 1999].

p53 is subject to acetylation in its C-terminus, by P300 and PCAF histone acetyltransferases [Liu et al.,1999] and these modifications are required for p53 to exert a proper transactivation activity. Other proteins can also sumoylate p53, by mechanisms not yet fully known [reviewed in Hollstein and Hainaut, 2010].

After various post translational modifications, p53 becomes a fully operative transcription factor, capable of positively and negatively transregulating genes that modulate cell cycle arrest or cell death (figure 3), while p53 is also able by itself to recruit proteins required during DNA repair [Rubbi and Milner, 2003]. The main p53 dependent pro-apoptotic genes are Bax, PUMA and Noxa [Fei et al., 2002]. The only p53 induced CKI is p21.

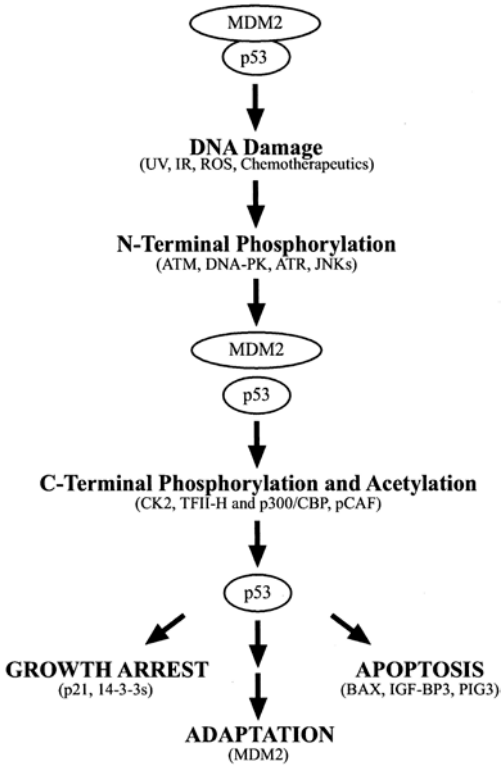


Figure 3 [from Cuddihy and Bristow, 2004]. p53 activation and stabilization allow it to function, leading to growth arrest and apoptosis. This activation can be reverted by destabilization of p53, mainly via Mdm2.

2.2 - Mdm2 and MdmX

The main controller of the halflife of p53 is the Mdm2 (*Murine Double Minute)* ubiquitin ligase and its homolog MdmX (in mouse; Hdm2 and HdmX are the human orthologs), which have been shown to act in both independent and interdependent ways [reviewed in Marine and Jochemsen, 2004].

Mdm2 is a member of the RING finger family of E3 ubiquitin ligases. Its RING finger binds directly to E2 ubiquitin-conjugating enzymes to promote ubiquitination of target proteins [reviewed in Sun, 2003], among which is p53. Mdm2 is also able to block p53 transactivational activity [Momand et al., 1992].

The Mdm2 gene transcription is dependent on two promoters: a weaker one that is constitutively active, and a strong one that is only triggered by p53 transactivation. This gives rise to a feedback loop, that keeps p53 protein level oscillating after genotoxic stimuli, while it stays low in the absence of an activation signal [Haupt et al., 1997 ; Kubbutat et al., 1997].

Mdm2 is subject to a variety of post-translational modifications [reviewed in Meek and Knippschild, 2003] that can modulate its ability to influence p53 activity: for example, phosphorylation of serine 395 by ATM inhibits the ability of Mdm2 to mediate p53 degradation and nuclear export [Khosravi et al., 1999]. Mdm2 can also act as an E3 ligase on itself, resulting in auto-ubiquitination and proteasomal degradation [Fang et al., 2000].

MdmX (also called Mdm4) [reviewed in Marine and Jochemsen, 2005] is an Mdm family member, of which the function is poorly understood, though it is able to bind directly to p53 and inhibit its transcriptional activity [Shvarts et al., 1996].

There is evidence for an interplay between Mdm2 and MdmX, since they can interact via their RING finger domains and form complexes [Tanimura et al., 1999].

Another important mechanism of countering the inhibition of p53 occurs through the interaction of the ARF protein ($p14^{ARF}$ in mouse and $p19^{ARF}$ in human) with Mdm2

[Pomerantz et al., 1998]. This interaction results in decreased Mdm2 binding to p53 and, consequently, to increased p53 activity [Zhang et al., 1998 ; Kamijo et al., 1998 ; Honda et al., 1999]. ARF can also bind to MdmX, leaving Mdm2 free and thus enabling it to counter p53 activation [Jackson et al., 2001].

3 - The Ubiquitin-Proteasome system (UPS)

The ubiquitin proteolytic pathway [reviewed in Ciechanover, 2005] plays a crucial role in the degradation of most cellular proteins. This influences regulation of the cell cycle and division, involvement in the cellular response to stress and DNA repair.

Ubiquitin-proteasome mediated proteolysis of the target substrate occurs in two discrete steps: covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the targeted protein by the 26S proteasome complex with recycling of free and reusable ubiquitin.

Initially, the ubiquitin activating enzyme E1 recruits and activates one free molecule of ubiquitin. Then one of the several ubiquitin conjugating enzymes E2 transfers the activated ubiquitin to the substrate, that is specifically bound to a protein of the E3 class. E3s provide substrate specificity and also catalyze the last step in the conjugation process, which is covalent attachment of ubiquitin to the substrate. The polyubiquitin chain serves as a marker for recognition by the 26S proteasome [reviewed in Ciechanover and Iwai, 2004].

The 26S proteasome is a complex that specifically degrades proteins in an ATP dependent manner: the catalytic unit of the protease is the 20S proteasome core. Two 19S cap complexes associate with the 20S cylindrical structure to generate the 26S proteasome (figure 4). These 19S complexes are regulatory subunits that determine substrate specificity and allow control [Glickman et al., 1998]. The process of recognition as a substrate for UPS degradation can be constitutive for some proteins, while for others

it requires post-translational modifications such as phosphorylation [reviewed in Ardley and Robinson, 2005].

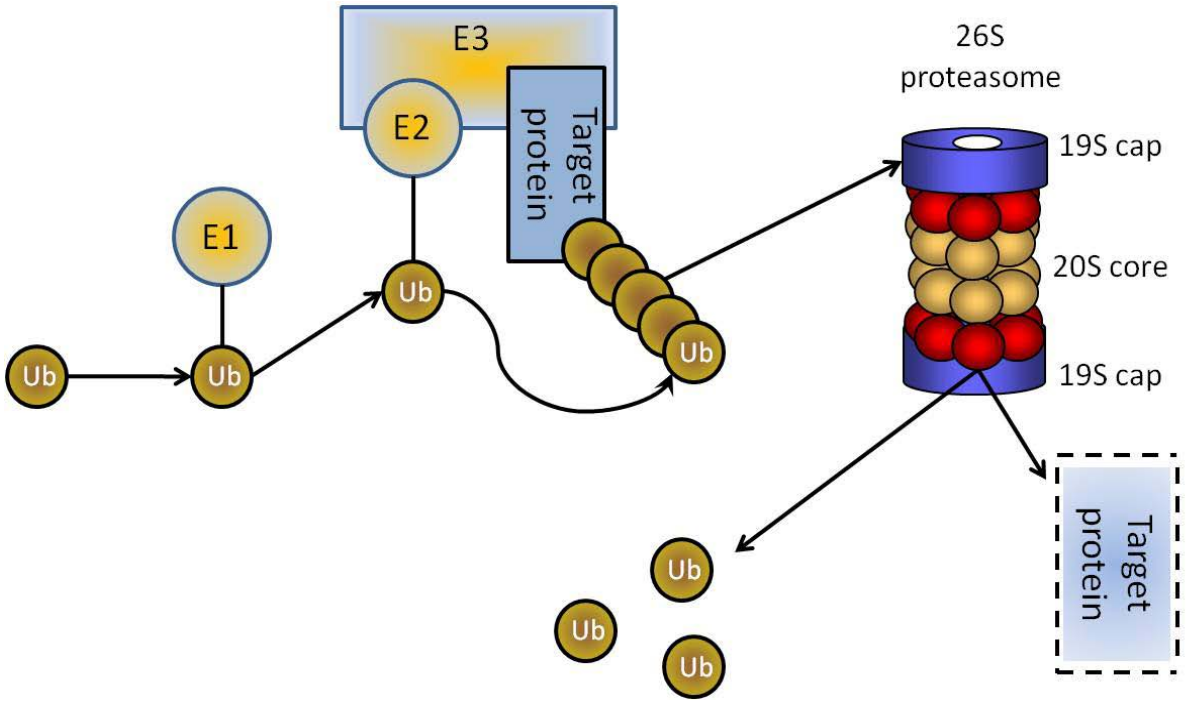


Figure 4. Scheme of the UPS mediated degradation: E1-E2-E3 mediate the polyubiquitination of an eligible target protein, which is then recognized by the 19S proteasome cap. The protease activity of the 20S core subunit degrades the target and regenerates free ubiquitin units.

Two main E3 enzyme complexes are known to regulate cell cycle progression: the anaphase-promoting complex or cyclosome (APC/C) and the Skp1/Cullin1/F-Box complex (SCF).

The APC/C [reviewed in Hershko et al., 1999 ; Simpson-Lavy et al., 2010] consists of twelve subunits and its composition varies through the cell cycle, as proteins like Cdc20 or Cdh1 have cell cycle phase related functions. In particular, APC/C^{Cdc20} is required for the transition through the spindle assembly checkpoint during M phase: when the aligment of chromosomes is completed, APC/C^{Cdc20} is activated and it degrades securin and cyclinB, leading both to completion and exit from mitosis [Sudakin et al., 1995]. In contrast,

APC/C^{Cdh1} is responsible for keeping mitotic signals low at the start of G1 phase and it has been implicated in endoreplication, where its activity is required to prevent cytokinesis [Garcia-Higuera et al., 2008].

Given that it targets G1 cyclins and CKIs for ubiquitination [Skowyra et al., 1997], the E3 ubiquitin ligase complex SCF initially was thought to regulate primarily G1/S progression. However, subsequently this complex was found to play important roles also during other phases of the cell cycle, depending on its subunit composition [reviewed in Nakayama and Nakayama, 2005]. The SCF consists of the invariable components Skp1, Cul1 and Rbx1 and a variable component, known as F-box protein, that binds to Skp1 through its F-box motif and is responsible for substrate recognition [Deshaies, 1999]. More than 70 F-box proteins have been identified in humans, amongst which are Fbw7, Skp2, βTrCP. This large number of F-box proteins provides the basis for multiple substrate-specific ubiquitination pathways. Different F-box proteins have been implicated in degradation of mitogenic transducers (Fbw7), degradation of CKIs (Skp2), regulation of APC/C (βTrCP). Like for APC/C, also for the SCF complex the binding to different subunits is cell cycle phase dependent, and indeed these two systems crossregulate each other depending on the cell cycle stage [reviewed in Vodermaier, 2004].

3.1 - Skp2

The F-box protein *S phase kinase-associated protein 2* (Skp2) has a core role in the cell cycle regulation, since it enhances progression through G1 phase. This is mediated by the ubiquitination of p27, subsequent to its phosphorylation on threonine-187 by the CyclinE–Cdk2 complex [Vlach et al., 1997]. In addition to p27, other cell cycle regulators have been implicated as potential substrates of Skp2. These proteins include the CKIs p21 and p57,

the pocket protein p130, CyclinE, the E2F-1 factor, the APC subunit Cdt1 and others [reviewed in Nakayama and Nakayama, 2005] (figure 5).

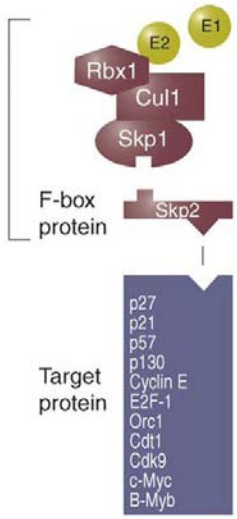


Figure 5 [modified from Nakayama and Nakayama, 2005]. The SCF^{Skp2} complex and its main targets

In addition to cell cycle regulation, Skp2 has been implicated in transcriptional regulation, by participating in the binding between p53 and its coactivator complex p300/CBP. Disrupting this interaction results in insufficent p53 acetylation, leading to decreased p53 stability [Kitagawa et al., 2008]. Skp2 can also modulate the c-Myc transcription factor activity, since these respond to ubiquitination with a transient increase in transactivational efficiency, followed by proteasomal degradation [Von der Lehr et al., 2003 ; Kim et al., 2003].

For a functional analysis of *Skp2,* knockout mice were generated [Nakayama et al., 2000]. *$Skp2^{-/-}$* mice have a phenotype comprising reduced body size and, as shown for fibroblasts and hepatocytes, endoreplication.

The generation of *Skp2/p27* double knockout mice [Nakayama et al., 2004] allowed to verify the relationship between these two proteins, with the result that some of the

phenotypes that occurred after *Skp2* removal are rescued, so that accumulation of p27 can be considered to be responsible for reduced body weight and endoreplication.

3.2 - Skp2 and p21

Skp2 has been previously implicated in the degradation of p21 [Bornstein et al., 2003], but the biological relationship between p21 and Skp2 has not yet been clarified. Indeed *in vitro* assays displayed evidence for a Skp2 mediated ubiquitination of p21 [Bornstein et al., 2003], while different cellular models are contradictory about this mechanism. For instance, *Skp2* gene amplification has been shown to promote ubiquitination and degradation of p21 in the context of hepatocellular carcinoma cell lines [Calvisi et al., 2009] while, in other experimental systems, Skp2 has been shown to be dispensable for p21 degradation: for example in HeLa cells, removal of *Skp2* leads to stabilization of p27, but not of p21 [Zhang et al., 2009]. Similarly in fibroblasts, p21 degradation after UV irradiation occurs in a Skp2-independent manner [Stuart and Wang, 2009].

4 - Ubiquitin independent proteasomal degradation

In addition to being part of the UPS, the proteasome alone can degrade proteins without prior ubiquitination. This mechanism is in part dependent on the structure of the 20S proteasome core [reviewed in Jariel-Encontre et al., 2008]. The 20S proteasome is a cylinder-shaped structure composed of 28 subunits distributed in 4 stacked rings. The two inner rings are identical and each one is composed of 7 different β subunits. These two rings define a central cavity that contains the catalytic sites. The two outer rings are also identical and made up of seven different α subunits. These rings allow the association of the 20S proteasome with a variety of regulatory complexes (among which is the 19S subunit of the proteasome) and control the translocation of substrates into the catalytic chamber through a narrow central orifice. In the basic 20S proteasome configuration, this

opening is gated by mobile extensions of the α subunits. However, various proteins are capable of changing the organization of these extensions to gain access to the proteolytic chamber. This leads to proteolysis of the substrates, that can constitutively elicit a proteasomal reconfiguration, without need for a ubiquitin conjugation (figure 6).

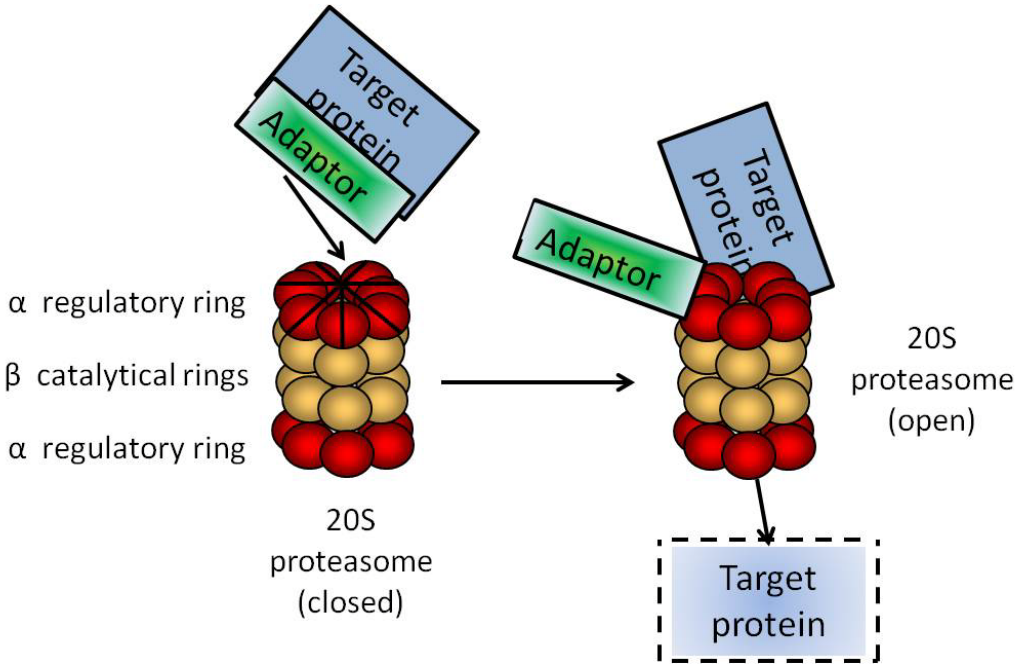


Figure 6. Scheme of the ubiquitin-independent proteasomal degradation: the 20S proteasome can bind and degradate a multitude of proteins that are able to open the regulatory α rings themselves, or with the aid of adaptors.

5 - p21

p21 was discovered in different cellular contexts by different groups.

$p21^{Cip1}$ (Cdk inhibitory protein 1) was first discovered as a cell cycle inhibitor, being a component of a quaternary complex that consists of cyclinD1, a Cdk and the proliferating cell nuclear antigen (PCNA) in normal human fibroblasts [Xiong et al., 1992]. $p21^{Waf1}$ (wild-type p53 activated factor) was discovered for being a direct target of the p53 tumor suppressor, as it mediates p53-dependent cell cycle arrest in response to DNA damage [el-Deiry et al., 1993]. Upregulation of Sdi1 (senescent cell-derived inhibitor 1), later known

to be $p21^{Sdi1}$, was characterized during a screening for senescence-related factors [Noda et al., 1994].

p21 acts during most phases of the cell cycle. During G1 phase, association of p21 with cyclin D-Cdk4/6 inhibits pRb phosphorylation and induces cell cycle arrest [Harper et al., 1995]. p21 can also directly inhibit E2F [Afshari et al., 1996 ; Delavaine and LaThangue, 1999], thereby countering progression through G1 phase and leading to cellular senescence. In S phase, p21 can bind PCNA [Oku et al., 1998], on a site that overlaps with the polδ and RFC interaction sites, and this association switches the function of the replication complex, from DNA synthesis to DNA repair, slowing down completion of S phase [Waga et al., 1994]. During G2 phase, p21 inhibits both CyclinB1-Cdk1 and CyclinA-Cdk1/2 complexes, leading to cell cycle arrest [reviewed in Taylor and Stark, 2001]. p21 also cooperates with 14-3-3σ, a p53 target which normally sequesters CyclinB1-Cdk1 complex in the cytoplasm, to impede the G2/M transition [Chan et al., 2000].

Since p21 is also present in active CyclinD-Cdk4 complexes [Zhang et al., 1994], p21 does not only act as a cell cycle inhibitor. In fact, p21 stabilizes and promotes active Cyclin-Cdk complex formation in a dose-dependent manner [LaBaer et al., 1997], suggesting that p21 can also promote normal cell cycle progression.

p21 is also important for the regulation of cell death, however both anti- and pro-apoptotic activities have been demonstrated [reviewed in Gartel and Tyner, 2002].

While under some experimental conditions overexpression of p21 has been shown to promote apoptosis, for example in thymocytes after ionizing radiation [Fotedar et al., 1999] and in p53-deficient mammary tumor cells [Shibata et al., 2001], endogenous p21 has been demonstrated to have also an antiapoptotic role in different experimental systems. For instance in human cancer cell lines treated with DNA damaging agents it has been shown that, instead of apoptosis, they undergo cell cycle arrest mediated by p21 [Zhang et

al., 2009]. Moreover, repression of the p21 promoter by c-Jun leads to p53 dependent apoptosis after UV irradiation [Shaulian et al., 2000]. A further example of p21-mediated antiapoptotic activity has been demonstrated in human colon cancer cells after Adriamycin-induced apoptosis [Bunz et al., 1999].

p21 is regulated at multiple levels, with crucial control points in its transcription and post-translational degradation.

Transcription of p21 (figure 7) is mostly dependent on checkpoint activation, as the p21 promoter contains two highly conserved p53 responsive elements. In addition, also the two p53 homologues, p63 and p73, have been shown to transactivate p21 [reviewed in Jung et al., 2010]. Independent of checkpoint activation, several transcription factors activate p21 expression. Indeed, responsive elements for several transcription factors are present in the proximal p21 promoter, including six Sp1/Sp3 binding sites. p21 transcription can also be repressed by some factors [reviewed in Gartel and Radhakrishnan, 2005], amongst which c-Myc, a bHLH transcription factor, is the most exhaustively described [Gartel et al.,2001].

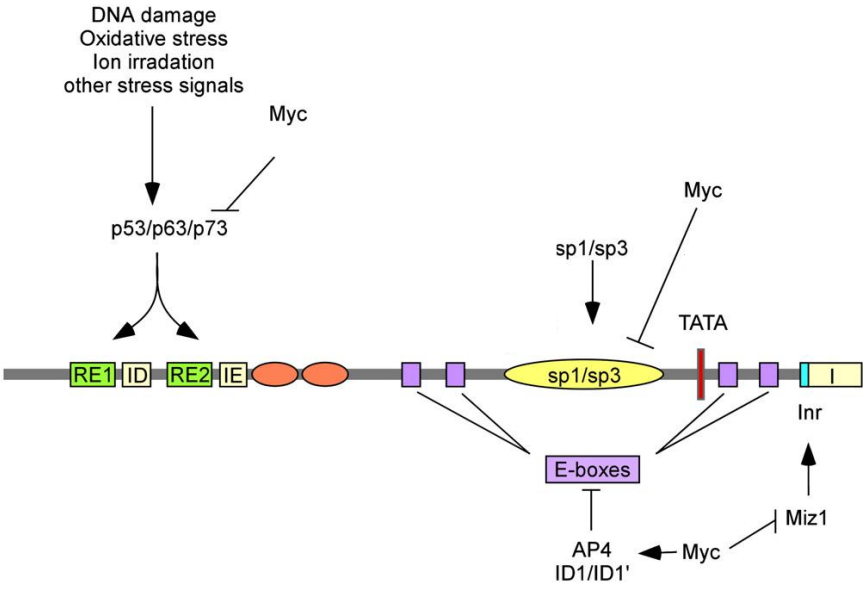


Figure 7 [modified from Jung et al., 2010]. Scheme of the main p21 promoter, showing influences by the p53 family, the Myc family and sp1/sp3 factors.

In addition to transcriptional regulation of p21, this CKI is also regulated post-translationally by the proteasome, by both ubiquitin-dependent and independent proteasomal degradation [reviewed in Jung et al., 2010].

Regarding the UPS-mediated degradation, several cell cycle-related E3 ligases target p21 at different stages of the cell cycle. During G1/S phase, Cdk2 mediated phosphorylation at serine 130 allows p21 to be recognized by SCF^{Skp2}, which then promotes polyubiquitination and, subsequently, degradation of p21 by the proteasome [Bornstein et al., 2003]. During G2/M phase p21 is targeted by APC/C^{Cdc20} and also destroyed by the UPS [reviewed in Simpson-Lavy et al., 2010].

While elimination of ubiquitination sites of p21 can prevent its degradation [Bloom et al., 2003], there is increasing evidence that p21 can be degraded by the proteasome also in an ubiquitin-independent manner [Chen et al., 2004]. Initially it was observed that p21 can interact with the α-subunits of the 20S complex, thereby being degraded directly by the proteasome [Touitou et al., 2001]. Different proteins were subsequently shown to influence ubiquitin-independent degradation of p21. p21 can be stabilized by the formation of CyclinD1–p21 complexes, which prevent p21 from this 20S proteasomal degradation [Coleman et al., 2003]. Similarly, Hsp90 binds to *de novo* synthesized p21 protein and prevents its degradation [Jascur et al., 2005]. On the other side, unlike conventional ubiquitin E3 ligases, Mdm2 promotes p21 turnover independently of its ubiquitin ligase activity [Jin et al., 2003]. In fact, recent evidence has shown that Mdm2 induces a conformational change in p21 that facilitates proteasome binding [Xu et al., 2010]. MdmX as well can promote p21 proteasomal degradation, both in cooperation with, and independently from, its homolog Mdm2 [Jin et al., 2008], by an unknown mechanism. These data indicate that p21 is subject to ubiquitin-dependent and independent degradation, but how these two pathways together regulate p21 stability remains to be determined.

6 - Experimental model and unpublished data

The P5 cerebellum is a convenient *in vivo* model to study the regulation of p21. It is precisely layered and each layer comprises cells with a particular differentiation status [Altman and Bayer, 1997]. The external granular layer (EGL) is divided into a mitotic zone (MZ) and a post mitotic zone (PMZ), the internal granular layer (IGL) is subdivided into a postmigratory zone and a zone which contains differentiated granular cells. Between the granular layers is the Purkinje cell layer (PCL), which contains cells of a different lineage, that are in an advanced maturation stage.

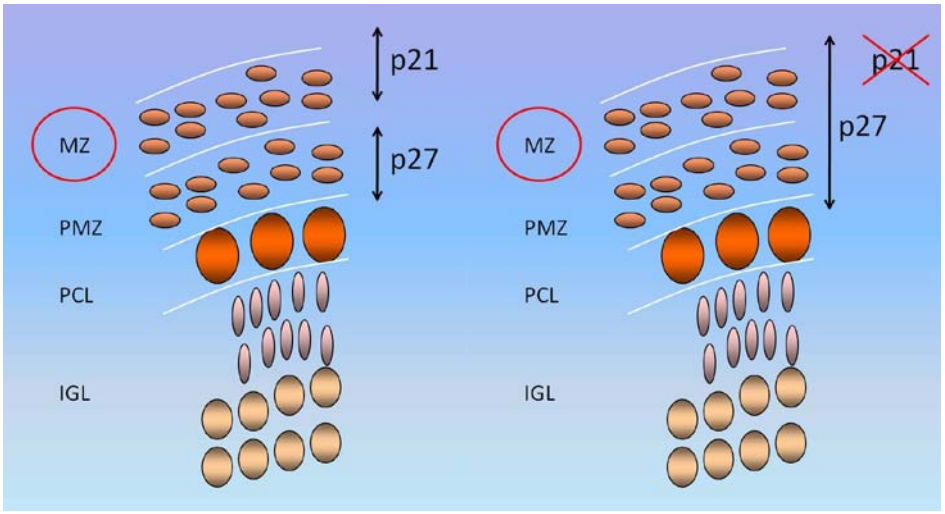
In the mitotic zone of the external granular layer lie the neuroblasts, which are neuronal precursors, not yet differentiated and still active in the cell cycle. As the neuroblasts cease to proliferate they express p27 [Miyazawa et al., 2000], permanently exit the cell cycle and migrate into the postmitotic zone, where they begin to express differentiation markers. During the continuation of this maturation process, they migrate through the Purkinje cell layer and stop in the postmigratory zone of the internal granular layer, where they carry out their differentiation programs and eventually become neurons.

The mitotic neuroblasts respond to ionizing irradiation by activating the p53 system, which leads to both pro- and anti- apoptotic responses [Herzog et al., 2002]. The outcome of irradiation can either be the stabilization of p21, linked with cellular survival, or the failure to stabilize p21 and the concomitant activation of the apoptotic system. Instead, after the p27 mediated cell cycle exit, cells in the postmitotic zone are no longer responsive to DNA damage.

Skp2 expression is known to be associated with both physiological and aberrant cell proliferation [reviewed in Hershko, 2008]. Consistent with this observation, at P5 the Skp2 protein is at its maximal expression in a wild type mouse cerebellum [Herzog, personal

communication], as this developmental stage is characterized by intense proliferation within the mitotic zone.

Provided p21 was degraded by Skp2, more p21 would be expected in the mitotic zone of *Skp2* $^{-/-}$ mice after irradiation, as compared to wild-types. Instead, previous data showed that p21 is strongly reduced in the mitotic zone of *Skp2* $^{-/-}$ mice [Bürkle and Herzog, unpublished].



Scheme of an irradiated P5 cerebellum. Localization of p21 and p27 in a wild-type cerebellum is shown on the left, in a Skp2 $^{-/-}$ *cerebellum on the right.*

A histological analysis regarding p21 stabilization in mitotic neuroblasts of different cell cycle phases has been previously carried out [Herzog, personal communication]. To investigate G2 phase, phospho-Histone H3 (PH3) was used. It was shown that p21 colocalizes with PH3 in the cerebellum of wild-types and *Skp2* $^{-/-}$ mice, suggesting that p21 is stabilized in cells of G2 phase. Moreover p21 was not detectable in S phase cells neither in wild-types nor in *Skp2* $^{-/-}$ mice.

Aim of the project

p21 has a crucial relevance in the resistance to DNA damage-induced cell death. As different studies argue over the pro- or anti-apoptotic effect of p21, it seems that p21 acts as an anti-apoptotic factor following irradiation, by halting the progression into cell cycle and allowing DNA repair [reviewed in Jung et al., 2010]. Thus, it is important to characterize the mechanisms and factors that influence the regulation of p21 and possibly find a way to alter the system, leading to an increase of cell death.

To investigate the fate of p21 in an *in vivo* system, the murine cerebellum at the fifth postnatal day (P5) was used. A functional analysis for the role of *Skp2* in this context was carried out *in vivo*, using *Skp2* knockout mice [Nakayama et al., 2000].

The aim of this work is to understand the role of Skp2 for p21 degradation. Starting from the data regarding the reduction of p21 in the *Skp2* $^{-/-}$ mouse, an analysis was carried out comparing wild-type and *Skp2* $^{-/-}$ animals, evaluating cell cycle perturbations and transcriptional regulation. In particular post-translational degradation of p21 was investigated regarding intermediate promoting factors, like Mdm2 and Mdm4.

Materials and Methods

1 - Materials

1.1 - Machines

- Table centrifuge Universal 320R – Hettich, Germany
- Thermal cycler – Biorad, USA
- Cryostate – Leica, Germany
- Fluorescence microscope Axioplan – Carl Zeiss, Germany
- Confocal laser microscope – Biorad, USA
- Electrophoretic system for DNA analysis – Biorad, USA
- Electrophoretic system for protein analysis – Biorad, USA
- UV spectrophotometer – Applied biosystems, USA
- Beta counter TriCarb 2100 TR – Packard, USA
- Tissue chopper – McIlwain, USA

1.2 - Chemicals and consumables

- Cell culture media and supplements – Sigma Aldrich, USA and Gibco, USA
- Chemicals – Delchimica, Italy and Roth, Germany and Sigma Aldrich, USA
- Tissue-Tek OCT compound – Sakura Finetek, Japan
- Superfrost Plus microscope slides – Menzel Glassner, Germany
- Nitrocellulose membrane for Western blotting – Perkin Elmer, USA
- Amersham ECL films for chemiluminescence detection – GE Healthcare, USA

1.3 - Reagents and enzymes

- Restriction enzymes – Promega, USA

- DNA polymerases – Promega,USA and Fermentas, USA
- Other DNA and RNA modification enzymes – Fermentas, USA
- ^{33}Pα-UTP – Perkin Elmer, USA
- Proteinase K – Roth, Germany and Sigma Aldrich, USA
- Protein A/G plus Agarose – Santa Cruz, USA
- Blocking reagent for Western blotting – GE Healthcare, USA
- Powdered milk – Hipp, Germany

1.4 - Drugs

- Bortezomib – LCLabs, USA
- BrdU – Roth, Germany
- Lactacystin – Enzo Life Science, USA
- Nutlin-3 – Enzo Life Science, USA

1.5 - Oligonucleotides

All oligonucleotides are supplied by Eurofins MWG, Germany

- Skp2 genotyping oligonucleotides:
    - KN3: 5’ – AGAGTGGAAGAACCCAGGCAGGAC
    - KN4: 5’ – CCCGTGGAGGGAAAAAGAGGGACG
    - KN13: 5’ – GCATCGCCTTCTATCGCCTTCTTG
    - KN38: 5’ – TTCCCACCCCCACATCCAGTCATT
- p27 genotyping oligonucleotides:
    - mgK3: 5’ - TGGAACCCTGTGCCATCTCTAT

- mcK5: 5’ – GAGCAGACGCCCAAGAAGC
- neo1: 5’ - CCTTCTATCGCCTTCTTG

1.6 - Buffers

- ELB
  - 50mM Tris pH7,5
  - 250mM NaCl
  - 0,1% NP-40
  - 1mM EDTA pH8
  - 2mM PMSF (added fresh prior to use)
  - 5mM DTT (added fresh prior to use)
- BC100
  - 20mM Tris pH7,9
  - 100mM KCl
  - 20% v/v Glycerol
  - 2mM PMSF (added fresh prior to use)
  - 5mM DTT (added fresh prior to use)
- Laird lysis buffer
  - 200mM NaCl
  - 100mM Tris pH8,5
  - 5mM EDTA pH8
  - 0,2% SDS
  - 100µg/ml Proteinase K (added fresh prior to use)
- TE
  - 10mM Tris
  - 1mM EDTA
  - pH adjusted to 8
- TAE buffer
  - 40mM Tris
  - 20mM Acetic acid
  - 1mM EDTA
- 2x hybridization solution
  - 1,2M NaCl
  - 20mM Tris ph 7,5
  - 4µg/ml PVP
  - 20µg/ml BSA
  - 2mM EDTA
  - 0,2mg/ml Salmon sperm DNA

    - 20% w/v Dextran sulfate
    - 0,1mg/ml Total yeast RNA
    - 0,1mg/ml Yeast tRNA
- RNAse buffer
    - 0,5M NaCl
    - 10mM Tris pH 7,5
    - 1mM EDTA
- 20x SSC
    - 3M NaCl
    - 0,3M Sodium citrate
    - pH adjusted to 7
- 1M Phosphate buffer
    - 1M PO_4
    - pH adjusted to 7,2 by mixing Na_2HPO_4 and NaH_2PO_4
- Paraformaldehyde solution:
    - 4% w/v PFA
    - 10% v/v 1M phosphate buffer
- Citrate buffer
    - 10mM Sodium citrate
    - pH adusted to 6
- Borate buffer
    - 0,1M sodium tetraborate
    - pH adjusted to 8,5
- Bicarbonate balanced saline
    - 0,1M sodium bicarbonate
    - 150mM NaCl
    - 10mM HEPES
    - pH adjusted to 8,2
- 10x PBS
    - 1,3M NaCl
    - 70mM Na_2HPO_4
    - 30mM NaH_2PO_4
    - pH adusted to 7,2
- 10x TBS
    - 1,3M NaCl
    - 250mM Tris
    - 25mM KCl
    - pH adusted to 7,4
- 5x SDS buffer
    - 10% w/v SDS
    - 10mM Beta-mercapto ethanol
    - 20% v/v Glycerol
    - 0,2M Tris pH6,8

    - 0,05% w/v Bromophenolblue
- SDS-PAGE running buffer
    - 25mM Tris
    - 0,2M Glycin
    - 0,1% w/vSDS
- Western blot transfer buffer
    - 25mM Tris
    - 0,2M Glycin
    - 20% v/v Methanol
- ECL
    - 1,25 mM Luminol in 100mM Tris pH8,5
    - 0,7 mM p-coumaric acid in DMSO
    - 0,01% H_2O_2

1.7 - Antibodies

- p21 (F5) – Santa Cruz, USA
- p21 (C19) – Santa Cruz, USA
- p27 (C19) – Santa Cruz, USA
- Mdm2 (2A10) – kindly provided by dr. A.J. Levine
- MdmX (MX82) – Sigma Aldrich, USA
- Cdk1 (17) – Santa Cruz, USA
- Cdk4 (Ab-1 DCS-35) – Lab Vision, USA
- Phospho-histone H3 – Upstate, USA
- BrdU – Immunologicals Direct, USA
- Chx10 – Exalpha Biologicals, USA
- Zic1 - Rockland, USA
- Tag1 – DSHB, Iowa University
- HRP conjugated anti mouse IgG - GE Healthcare, USA
- HRP conjugated anti rabbit IgG - Chemicon Australia

- Cy2 conjugated anti rat IgG - Jackson Immunoresearch, USA
- Cy2 conjugated anti rabbit IgG - Jackson Immunoresearch, USA
- Cy3 conjugated anti rabbit IgG - Jackson Immunoresearch, USA
- Cy3 conjugated anti mouse IgG - Jackson Immunoresearch, USA
- Cy3 conjugated anti sheep IgG - Jackson Immunoresearch, USA
- Biotin–spacer conjugated anti mouse IgM+ IgG - Jackson Immunoresearch, USA
- Cy3 conjugated streptavidin – Molecular Probes, USA
- Cy2 conjugated anti mouse IgG2B - Invitrogen, USA
- Cy3 conjugated anti mouse IgG1 - Invitrogen, USA

1.8 - Animals

- C57Bl/6J mice – Charles River, USA
- *Skp2* heterozygous mice [Nakayama et al., 2000] – kindly provided by dr. K.I. Nakayama
- *p27* heterozygous mice [Fero et al., 1996] – Jackson Labs

2 - Methods

2.1 - Genotyping

2.1.1 - DNA preparation [Laird et al., 1991]

To perform genotyping, a small piece of tail (2mm long) is taken from mice. It is incubated in Laird lysis buffer at 56°C for 4 hours or overnight. When the digestion is complete, the DNA contained in the supernatant is precipitated by addition of 1 volume isopropanol. DNA

is pelleted by centrifugation, then washed with 70% ethanol and finally allowed to become almost dry. The DNA pellet is then resuspended in TE buffer for downstream applications.

2.1.2 - PCR

Genotyping is carried out by PCR. The general formula for PCR mastermixes is as follows:

- 1X TAQ buffer, already including $MgCl_2$
- 2µM each specific primers
- 250µM each dNTP
- 3u/100µl TAQ DNA polymerase
- Template DNA in variable amount

Skp2 genotyping PCR protocol

- Initial denaturation: 5 minutes at 94°C
- 30 cycles of:
    - denaturation: 30 seconds at 94°C
    - annealing: 30 seconds at 62°C
    - elongation: 90 seconds at 72°C
- final elongation: 5 minutes at 72°C

p27 genotyping PCR protocol

- Hot-start at 95°C
- Initial denaturation: 5 minutes at 95°C
- 4 cycles of:
    - denaturation: 30 seconds at 96°C
    - annealing: 30 seconds at 57°C

    - elongation: 2 minutes at 65°C
- 36 cycles of:
    - denaturation: 30 seconds at 93°C
    - annealing: 30 seconds at 57°C
    - elongation: 2 minutes at 65°C
- final elongation: 2 minutes at 65°C

2.1.3 - Electrophoresis of PCR products

The PCR products are analyzed by electrophoresis, using a 1% agarose gel in TAE buffer; ethidium bromide is included in the gel solution (0,4µg/ml).

2.2 - Preparation of mouse tissues for biochemical analyses

Mice are anesthesized by hypothermia. The head is cut, then the brain is exposed by performing a caudal-frontal cut through the skin and then the skull. The brain is removed and the cerebellum is dissected and cleaned of the meninges. Eyes are taken from the orbits and cleaned of the lacrimal glands and muscles. The cerebellar tissue and the eyes are then frozen using liquid nitrogen or dry ice and stored at -80°C for further analyses.

2.3 - Intracardiacal perfusion of mice for histological analyses

Mice are anesthesized by hypothermia, then a horizontal incision is made under the sternum, to expose the diaphragm; this muscle is cut and, by performing a lateral ascending cut, the chest is opened and can then be flipped aside. As the heart is exposed, an incision is made in the right atrium, to open the circulation, and a solution of 4% PFA is injected into the left ventricle: this procedure substitutes blood with PFA. After perfusion, the cerebellum and eyes are removed as described above and tissues are postfixed

overnight at 4°C in PFA solution. The following day PFA is removed and substituted by a solution of 20% w/v sucrose in PBS, in which tissues are incubated overnight at 4°C. This prevents water crystals formation and allows cryosectioning,. After sucrose incubation the tissue is immersed in TissueTek compound and placed on dry ice. This causes the compound to become solid, thus including the tissue. A block is obtained, which can be cut using a cryostate. The cryosections obtained this way are let dry at room temperature and then stored at -20°C for following immunohistological analyses.

2.4 - Immunofluorescent histological labelling

Sections are taken from -20°C storage and allowed to dry at room temperature. Unless otherwise stated, the buffer used for immunofluorescence in all washing steps (three subsequent washes) and for the dilution of blocking solution and antibodies is PBS-Triton 0,1%.

Sections are blocked for one hour at room temperature using a 1,5% horse serum solution, then the primary antobody is added, usually in a 1:100 dilution, and left for one hour at room temperature or overnight at 4°C. After a washing step, the secondary antibody is added, usually in a 1:200 dilution, and left one hour at room temperature. After a last washing step, a 1:1 PBS-glycerol solution is added as a preservative and the slides are mounted with coverslips.

Peculiar immunostainings:

2.4.1 - p21 immunostaining

An antigen retrieval step must be performed before commencing the staining. It is carried out by placing the slides in citrate buffer, which is then warmed up to nearly boiling temperature and kept heated for 10 minutes. Afterwards the sections are left inside the solution to slowly reach room temperature.

2.4.2 - BrdU labelling

Mice are injected Bromodeoxyuridine (10mg/kg) one hour prior to perfusion. BrdU is then detected by immunostaining on cryosections. To do this, sections are treated with 2M HCl at 37°C for 15 minutes, then pH is reverted to neutral by washing with a strong buffer (1M sodium tetraborate pH 8,5). Tissues are then processed for immunostaining.

2.4.3 - Biotinylated secondary antibody

This antibody is used to label mouse primary antibodies of the IgM and IgG class with a green fluorescent dye. The secondary antibody is diluted 1:100 in PBS-Triton 0,1% and incubated for one hour at room temperature. After a washing step, sections are washed in bicarbonate balanced saline, then they are incubated with Cy2-conjugated streptavidin, diluted 1:200 in balanced saline. After 30 minutes of incubation, sections are washed in balanced saline and then in PBS-Triton 0,1%.

2.5 - Preparation of protein samples and Western blotting

Tissues are taken from -80°C storage and homogenized in an appropriate amount of ELB buffer (100 µl for half cerebellum or 250 µl for one eye) by using a Wheaton tight stempel homogenizer. The lysate is cleared by centrifugation and the needed volume of 5xSDS buffer is added. Samples are then heat shocked by boiling for 3 minutes and by rapidly cooling them down on ice. These preparations are ready to be used in Western blotting and can be stored at -20°C for further analyses.

All protein samples are resolved using the SDS-PAGE technique prior to blotting. Western blotting is carried out by “wet” procedure and proteins are transferred on nitrocellulose membranes.

Unless otherwise stated, the general protocol for immunoblotting uses PBS-Tween 0,1% for all washing steps (three washes each) and as a dilution buffer for blocking reagent and antibodies. The membranes are blocked with a one hour incubation at room temperature in a 5% blocking reagent solution. The primary antibody is then incubated for one hour, usually in a 1:1000 dilution, in the presence of 10% horse serum. After a washing step the secondary antibody is added for one hour, usually in a 1:2000 dilution, also in the presence of 10% horse serum . After a final washing step, the membrane is treated for chemilumiscence detection, by applying ECL solution. After letting luminescence increase for one minute, the signal is detected by exposure of photographic films.

Peculiar western blotting protocols

2.5.1 - Mdm4

Membranes are blocked for one hour at room temperature with powdered milk, in a 10% w/v dilution made in TBS-Tween 0,2%. The primary antibody (MX-82) is diluted 1:1000 and incubated overnight at 4°C in the same solution. The secondary antibody is diluted 1:2000 in a 5% w/v milk solution made in TBS-Tween 0,1% and incubated one hour at room temperature.

2.5.2 - Mdm2

Blocking and incubations with both primary (2A10) and secondary antibodies are performed in a 5% milk solution in TBS-Tween 0,1%

2.6 - Coimmunoprecipitation

Tissues are homogenized in BC100 buffer by using a Wheaton tight stempel homogenizer. The lysate is then cleared by centrifugation. A volume corresponding to 10% of the total supernatant is taken for loading control. The remaining material is incubated overnight at

4°C with the chosen antibody for selective immunoprecipitation. Protein A/G plus agarose beads (20µl per precipitation) are washed in BC100 buffer and then incubated overnight at 4°C in BC100 buffer, added with 1,5% w/v BSA. The following day, beads are centrifuged and blocking solution is replaced with the immunoprecipitate. Binding between beads and antibodies is allowed at room temperature for two hours, after which the beads are washed in BC100 buffer and eventually mixed with an appropriate volume of SDS buffer and boiled to perform heat shock. Samples are then ready for SDS-PAGE.

2.7 - cDNA preparation from tissues

All disposables employed are RNAse free, while non disposable materials are previously cleaned with 0,1M NaOH and rinsed in DEPC treated water. All procedures are carried out on ice to minimize RNA degradation. Tissues are taken from -80°C storage and homogenized in TRIZOL by using a Wheaton loose stempel homogenizer. Chloroform is added in a ratio of 1:1 and samples are shaken to allow mixing. The material is then centrifuged to allow separation of the phases and the upper acqueous phase is saved. Isopropanol is added in a 1:1 ratio to precipitate nucleic acids, which are pelleted by centrifugation. The pellet is washed with 70% ethanol and briefly allowed to dry. Nucleic acids are then quantified using a UV spectrophotometer. 3µg of each sample are treated with RNAse free DNAse for 20 minutes, then heated to 60°C in the presence of 10 µM EDTA for 10 minutes, to inactivate the nuclease. An agarose gel is then run with 1µg material to assess the quality of the RNA. Reverse transcription is performed on 1µg RNA using an H-minus recombinant RNA-dependent DNA polymerase.

2.8 – Real-time RT-PCR

cDNA samples were submitted for analysis to the Cogentech company (Milan, Italy). Assays were performed using pre-designed TaqMan probes (Applied Biosystems),

following the ABI 7900HT sequence detection system and the ABI PRISM 7700 sequence detector analyser user's manual. Results were analysed with the SDS 2.1.1 software (Applied Biosystems) and ready-to-read data tables were obtained from the company.

2.9 - In situ hybridization

Cryosections are prepared and treated to ensure RNAse free conditions. Radiolabelled RNA probes are prepared from a pBluescript construct in the case of p27 and from a T3-T7 PCR product for p21. The template DNA is cut with the appropriate restriction enzyme, then denatured by heat shock. 0,5µg DNA is added to the RNA polymerase reaction, which contains radiolabelled ^{33}Pα-UTP.

In Vitro Transcription mastermix:

5x transciption buffer	4µl
100mM DTT	2µl
NTP mix minus UTP	1µl
RNAseOUT	1µl
template DNA	0,5µg/1µl
^{33}Pα-UTP	3,5 µl
T3 or T7 RNA polymerase	5u/1µl
RNAse free H_2O	to 20µl

The reaction proceeds for one hour at 37°C, after which 5 units of RNAse-free DNAse are added for 20 minutes to destroy the DNA template. The product is then precipitated at -80°C using the acetate-ethanol method. The material is centrifuged and washed and the RNA pellet is resuspended in TE-SDS 0,1%. A volume of 1% is taken from both the probe and the washes to perform a radioactivity assay, using a beta counter machine. This allows to assess the efficiency of transcription and to know the total amount of radiolabelled probe obtained. Usually, $4,5x10^6$ cpm/ml is the required level of radioactivity

in the hybridization reagent, which is composed of the appropriate amount of probe, plus 50% 2x-Hybridization solution, 50% formamide, 0,1% SDS. Prior to addition to the hybridization reagent, the probe is linearized by heating-cooling, while the 2xHyb is pre-heated at 60°C. The complete reagent is then added on top of pre-heated microscope slides, that are then covered with 60mm long coverslips and placed in a humid chamber soaked in a 1:1 4xSSC-formamide solution. The hybridization is carried out overnight at 56°C. The following day, sections are washed in a 1:1 4xSSC-formamide solution, then rinsed for 15 minutes in 2xSSC and finally treated with RNAseA (100 µg/ml dissolved fresh in RNAse buffer), to destroy unbound probe. Afterwards the slides are washed in a gradient of raising temperature and decreasing salt: the first wash is made in RNAse buffer at 50°C for 30 minutes, the second with 2xSSC at 60°C for 30 minutes, the third with 0,2xSSC at 65°C for one hour. The slides are finally dehydrated in an ethanol series (50% - 70% - 95% - 100%), with the addition of 0,3M ammonium acetate. After letting the slides dry, a hyperfilm sheet is exposed overnight directly on top of the slides, to make a preliminary detection of the signal. The following day, slides are dipped into photographic emulsion and left in a dry dark chamber, from 14 to 28 days, based on the signal intensity previously detected. After the desired number of days, slides are developed with D19 developer and photographic fixer (KODAK). If needed, sections can be counterstained with DAPI.

2.10 - Cerebellar granular cells culture

2cm round coverslips are coated overnight at room temperature in a solution of 0,1 mg/ml poly-L-lysine in a 1:1 boric acid 1,25% /sodium tetraborate 1,9% mixture. Cerebella are taken from P7-P8 mice and cleaned from the meninges. They are subsequently incubated for 15 minutes at 37°C in a 0.5% trypsin solution in Hank's balanced salt solution. Trypsin is then removed and cerebella are placed in a 0,5 mg/ml DNAse solution and dissociated

by 7 pipetting strokes through a P1000 tip. After letting the tissue bits precipitate, the supernatant is taken and mixed with an equal amount of 10% FBS medium for trypsin neutralization. Cells are centrifuged at 250g for 10 minutes and counted. $3x10^6$ cells are seeded in neurobasal medium, containing Glutamax, antibiotics and B27 supplement, on a 2.5 cm dish, containing a coated glass coverslip. After 30 minutes at 37°C the medium is replaced with complete neurobasal medium (see above) supplemented with 25mM KCl. Cells are cultured for 5 days and then fixed with 4% PFA to be used for immunostainings.

2.11 - Nuclear size quantification

To label cells in the postmitotic zone, a Tag1 immunostaining is performed, together with a DAPI staining to detect nuclei (figure 1a).

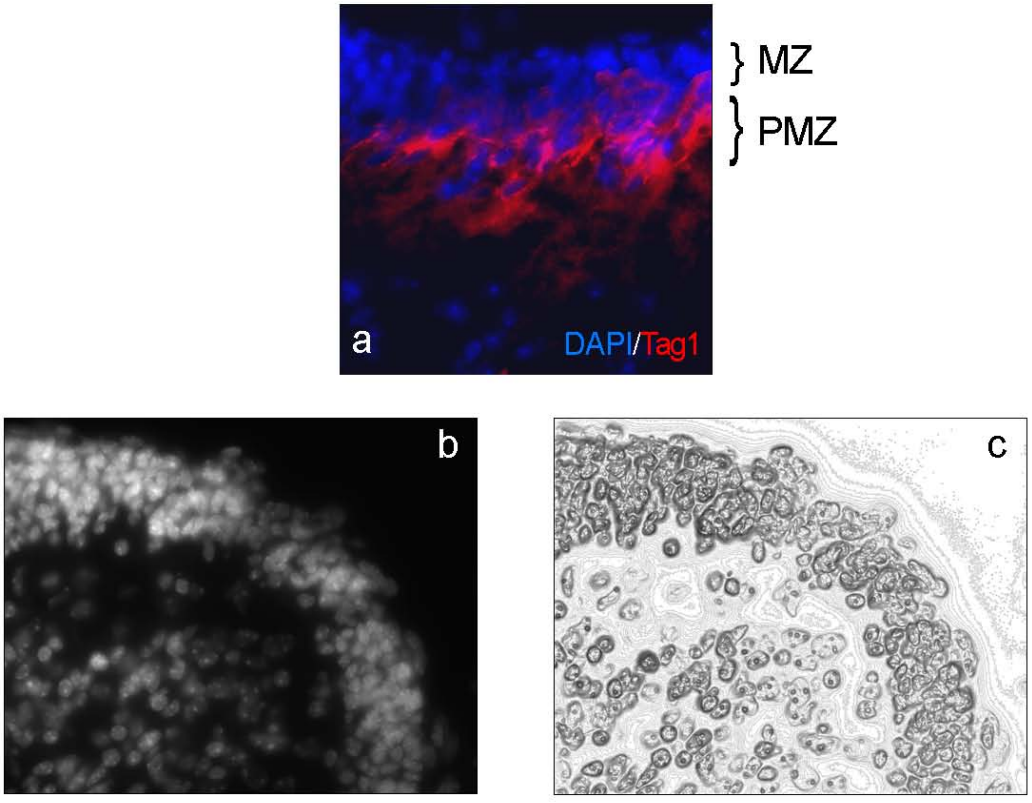


Figure 1. a) Cerebellar section stained with the anti-Tag-1 antibody, which labels the PMZ, and DAPI, which labels DNA.. b) Image of a cerebellar section stained with DAPI. c) Mask of the previous cerebellar section, obtained through the ImageJ software.

Nuclear size from Tag1 negative cells is measured using the ImageJ software (NIH). Starting from images of DAPI stainings (figure 1b), a “mask” is created to highlight the boundaries of the nuclei and discriminate adjacent cells (figure 1c). The perimeter of these shapes is measured using the program and the volume is then calculated assuming they had a spherical form.

2.12 - Cerebellar slice cultures for proteasome inhibition experiments

The cerebellum is taken from a P5 mouse (1 hour irradiated or non irradiated), cleaned of the meninges, and cut into 800 µm thick slices using a tissue chopper. Slices are then cultured in 24 wells multiwell plates for up to three hours. The culture medium is Neurobasal, with B27 supplements, Glutamax and antibiotics. Lactacystin is used in a final concentration of 20 µg/ml. At the needed time point, slices are either fixed using PFA or homogenized in ELB buffer.

2.13 – Animal treatments

Irradiation consists in administering 14 Gy of whole body irradiation. Perfusions and tissue explants are usually performed three hours after irradiation start.

Nutlin-3 and Bortezomib are administered by dorsal subcutaneous injection, 1 hour before irradiation. Nutlin-3 is used at 20 mg/kg, Bortezomib is employed at 10 µg/kg. BrdU was administered at 10 mg/kg by intraperitoneal injection 1 hour before perfusion.

Results

1 – p21 stabilization through cell cycle phases

1.1 - p21 is stabilized in G2 phase cells of the mitotic zone

Previously it was shown that p21 can colocalize with phospho-Histone H3 (PH3) in the cerebellum of wild-type mice and *Skp2* $^{-/-}$ mice as well [Herzog, personal communication], suggesting that p21 is stabilized during G2 phase. However, the PH3/p21 double staining could not be used to investigate M phase; after irradiation PH3 is dowregulated in M phase cells in an Atm-dependent manner [Xu et al., 2002] and the full nuclear staining of PH3 is not detectable anymore. In this context it must be noted that M phase cells represent only a 2,5% of all cells in the EGL at P10 [Fujita, 1967], so their quantitative impact should be negligible presumably also at P5.

During G2 phase, p21 binds to Cdk1 [reviewed in Taylor and Stark, 2001]. Thus, to further support that p21 is maintained in mitotic neuroblasts during G2 phase, a coimmunoprecipitation experiment between p21 and Cdk1 was carried out, both in wild-type and *Skp2* $^{-/-}$ cerebella. Cerebellar extracts were incubated with the anti-p21 antibody C-19 and subsequently Cdk1 was detected by western blotting (figure 1).

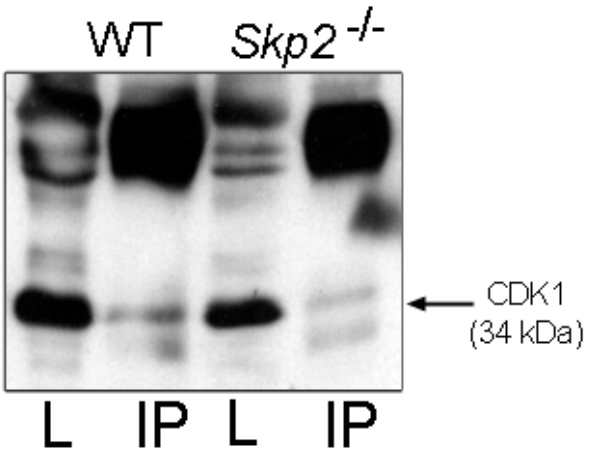


Figure 1. p21 interacts with Cdk1 in wild-type (WT) and Skp2 $^{-/-}$ cerebella. Western blot analysis of cerebellar protein extracts: 90% of each extract was immunoprecipitated with the anti-p21 antibody C-19 (IP lanes); 10% was loaded as control (L lanes). Western blot was carried out against Cdk1.

Since p21 binds to Cdk1 both in the wild-type and *Skp2* $^{-/-}$ cerebella, this result shows that p21 is stabilized in G2 phase irrespective of the *Skp2* status.

1.2 – G1 phase and endoreplication

Since no G1-specific antigens were available, identification of G1 phase cells in the cerebellum was not possible by histological methods. p21 has been shown to be stabilized during G1 and G2 phases in other experimental systems [Agarwal et al., 1995 ; Delia et al., 2003]. Thus, coimmunoprecipitation assays were carried out to look for interactions between p21 and G1 phase cyclins and Cdks: previous data showed that p21 cannot bind to cyclinE or Cdk2 in the cerebellum, however it was shown that p21 interacts with cyclinD1, Cdk4 and Cdk6 [Herzog, personal communication].

Based on these data, p21 was found to interact with Cdk4 (figure 2) both in wild-type and *Skp2* $^{-/-}$ mice.

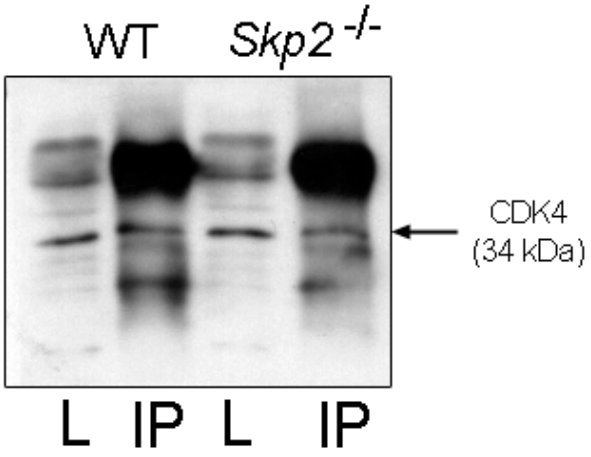


Figure 2. p21 interacts with Cdk4 in wild-type (WT) and Skp2 $^{-/-}$ cerebella. Western blot analysis of cerebellar protein extracts: 90% of each extract was immunoprecipitated with the anti-p21 antibody C-19 (IP lanes); 10% was loaded as control (L lanes). Western blot was carried out against Cdk4.

Although this interaction suggests that p21 can be maintained during G1 phase, regardless of Skp2, it must be considered that Cdk4 does not only bind p21 during G1 phase, but also during G2 [Gabrielli et al., 1999]. In addition, it must be noted that G1 phase comprises 45% of cells in the EGL at P10, while G2 cells are only 10,5% [Fujita, 1967]. Thus, the strong diminution of p21 in *Skp2*$^{-/-}$ cerebella can be attributed mainly to G1 phase cells.

It was shown that, in the *Skp2* $^{-/-}$ mouse, hepatocytes and embryonic fibroblasts endoreplicate [Nakayama et al., 2000]. While generally endoreplication is characterized by the omission of cytokinesis, so that a cell can complete one cell cycle without splitting into daughter cells [reviewed in Edgar and Orr-Weaver, 2001], there are several forms of endoreplication. In particular the G1 phase can be bypassed and this could cause a lack of G1 checkpoint activation, leading to reduced p21 levels.

Endoreplicated cells display two hallmarks: the presence of additional genetic material, and consequently an increase in nuclear size, and the accumulation of supernumerary centrosomes [reviewed in Edgar and Orr-Weaver, 2001].

The presence of additional genetic material was previously investigated by a FACS analysis on nuclei extracted from whole cerebella of wild-type and *Skp2* $^{-/-}$ mice [Ulrich and Herzog, unpublished]. No difference was evident, as the number of cells containing a 4N or more DNA content were similar in both populations.

While this analysis demonstrated no difference between wild-type and *Skp2*$^{-/-}$, it is possible that endoreplication occurred only in a subpopulation of cerebellar cells and hence it was obscured by the normal replication of other cell types.

1.2.1 – Nuclear size is not altered in *Skp2* $^{-/-}$ neuroblasts

To restrict the analysis to mitotic cells of the EGL, sections of cerebellar tissue were immunostained with the TAG1 antibody, that labels the postmitotic zone, and DAPI, that stains the nuclei. Using the ImageJ software (NIH), this method allowed to quantify the nuclear size of mitotic neuroblasts. Consistent with the pre-existing data, this analysis showed no difference between the nuclear sizes of cells from wild-type and *Skp2* $^{-/-}$ mice (figure 3).

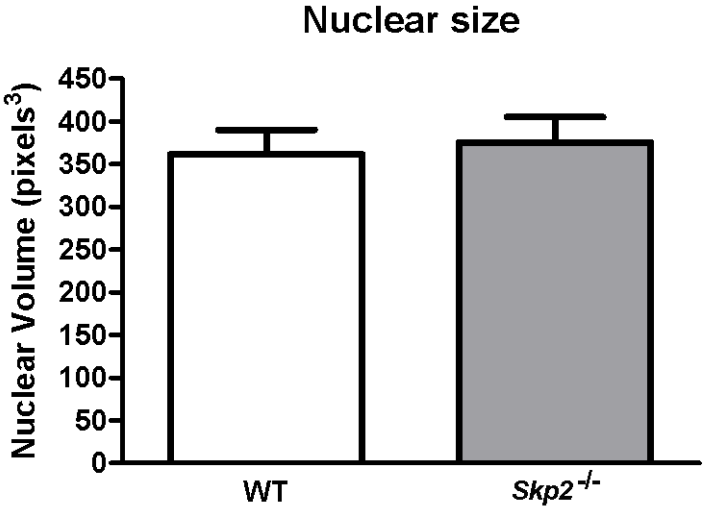


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Figure 3. Nuclear volume of mitotic neuroblasts is not altered in the absence of Skp2 $^{-/-}$*. Bar graph comparing nuclear volumes between wild-type (WT) and Skp2* $^{-/-}$ *mitotic neuroblasts. Volume is arbitrarily quantified as cubic pixels on images taken with the same zoom factor.*

1.2.2 – Centrosomes are not overduplicated in the absence of *Skp2*

To investigate the second hallmark of endoreplication, the presence of supernumerary centrosomes was analyzed. In *Skp2* $^{-/-}$ embryonic fibroblasts, centrosomes have been demonstrated to be amplified [Nakayama et al., 2000]. Therefore, a centrosome analysis was performed on primary cultures of cerebellar granular cells. The cultures, obtained from either wild-type or *Skp2* $^{-/-}$ cerebella, were allowed to grow for 3 to 5 days, then fixed and stained in a triple immunofluorescence for gamma-Tubulin (γ-Tubulin), which is a specific marker for the centrosomes, PH3, that labels cells in G2/M phase, and DAPI, that labels DNA. Comparison between representative fields, taken from wild-type and *Skp2* $^{-/-}$ cultures, showed no relevant difference; supernumerary centrosomes were very rare, in cells of both genotypes (figure 4).

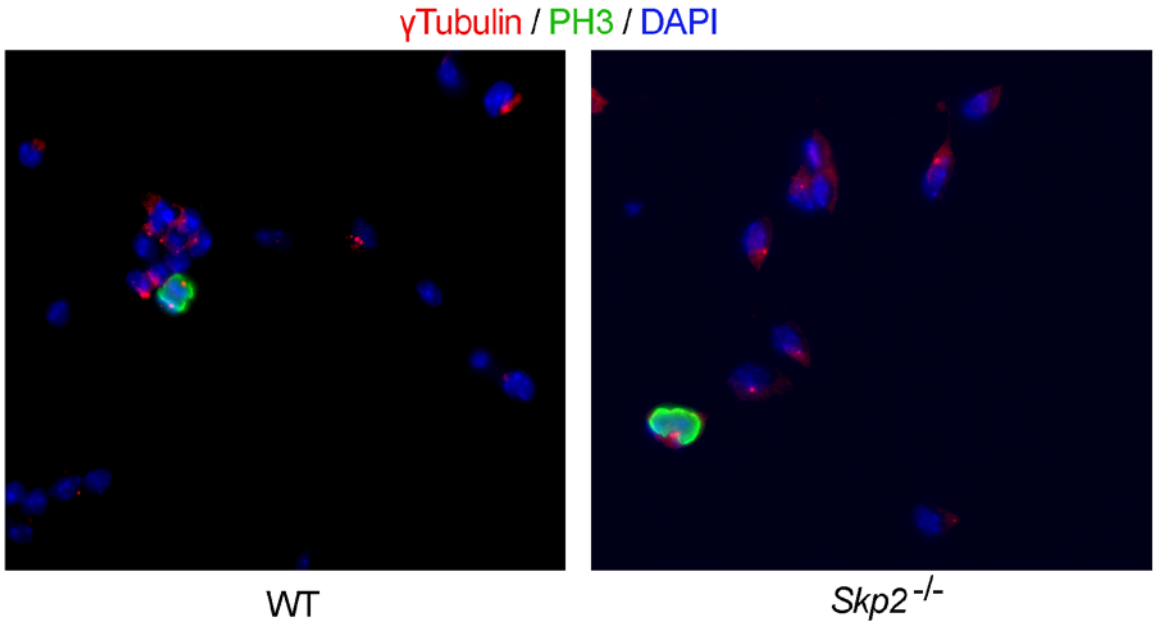

Figure 4. Skp2 $^{-/-}$ *cerebellar granular cells do not overduplicate centrosomes. Comparison between representative fields of cerebellar granular cells cultures obtained from wild-type (WT) and Skp2* $^{-/-}$ *mice. A triple immunostaining was used to label nuclei (DAPI-blue), G2/M phase cells (PH3-green) and centrosomes (γ-Tubulin-red).*

Since the hallmarks of endoreplication were found to be absent in the context of *Skp2* $^{-/-}$ cerebella, it is possible to exclude this alteration of the cell cycle as a cause for p21 reduction.

2 – p21/p27 interplay

Previously it was shown for other cell types that p27 is upregulated in the absence of *Skp2* [Nakayama et al., 2000]. Similarly, in cerebellar neuroblasts from *Skp2* $^{-/-}$ mice p27 levels are higher as compared to wild-types. In wild-type cerebella p21 and p27 are mutually exclusive. While p21 is in the mitotic zone, p27 is in the postmitotic zone. In contrast, p27 accumulates ectopically in the mitotic zone of *Skp2* $^{-/-}$ mice (figure 5), in cells of G2 phase (that are doublestained for p21 and p27 in the *Skp2* $^{-/-}$) and, presumably, also in G1 phase cells.

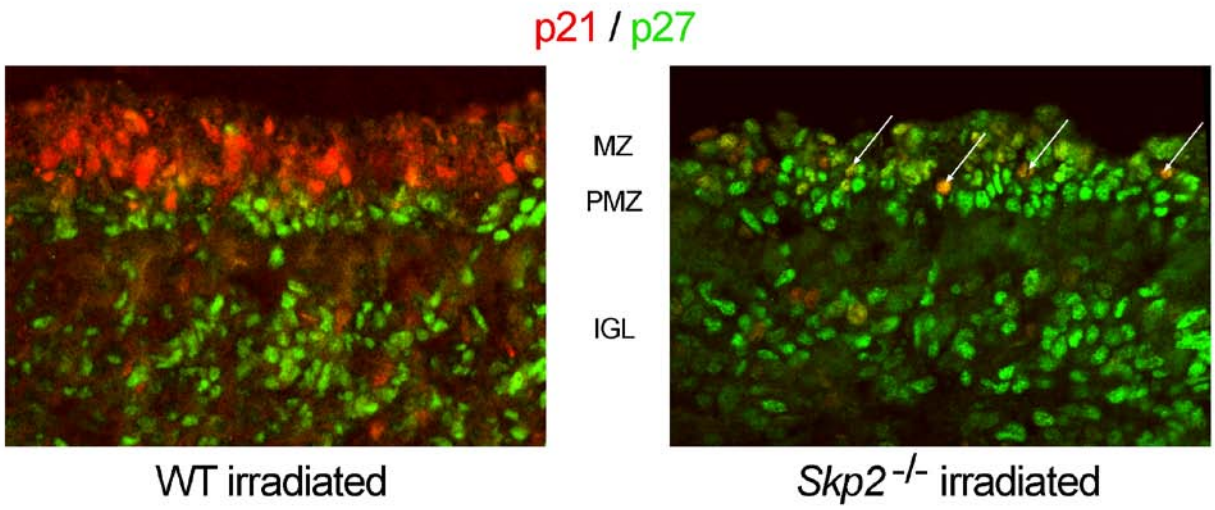

Figure 5. p21 and p27 localization in wild-type (WT) and Skp2 $^{-/-}$ *irradiated cerebella. Cerebellar sections were immunostained for p21 (red) and p27 (green). The mitotic zone (MZ), postmitotic zone (PMZ) and internal granular layer (IGL) are indicated. Arrows indicate p21/p27 double-positive cells (yellow cells) in the Skp2* $^{-/-}$.

Apparently the *p21* and *p27* systems are able to communicate with each other on multiple levels, being able to exert the same functions [Cheng et al.,1999 ; Jirawatnotai et al.,2003 ; Holsberger et al., 2005] and crossregulating each other [Kwon et al., 2002]. Therefore, this reciprocal regulation was investigated in the context of *Skp2* elimination.

2.1 – Transcription analysis

2.1.1 – p21 transcription

The p21 promoter is subject to regulation by many positive and negative factors, among which p53 is the main activator after DNA damage [Gartel and Radhakrishnan, 2005]. Indeed, Skp2 has been shown to be involved in the regulation of some transcription factors, in particular the p300/CBP complex and the c-Myc transcription factor from the Myc family, that are known to be involved in p21 mRNA expression. Skp2 can bind to the p300 acetyltransferase, which is an essential activator of p53 [Kitagawa et al., 2008], so in the absence of Skp2 the complex might be lacking a component, being consequently less active. Skp2 is also known to promote the ubiquitination of the c-Myc transcription factor [Kim et al., 2003 ; von der Lehr et al., 2003], that directly binds to the p21 promoter [Seoane et al., 2002]; the ubiquitinated form of c-myc has an increased transactivational activity, before it is degraded by the proteasome, so the lack of Skp2 might lead to an insufficient c-myc activation of the p21 promoter and hence to reduced p21 mRNA levels.

2.1.1.1 – p21 mRNA levels are not decreased in *Skp2* $^{-/-}$ cerebella

To investigate p21 mRNA quantities, cerebella from wild-type or *Skp2* $^{-/-}$ mice were taken, either without treatment or 3 hours after irradiation. RNA was extracted from 4 cerebella for each experimental condition, then cDNA was synthesized and samples were submitted to real time RT-PCR analysis.

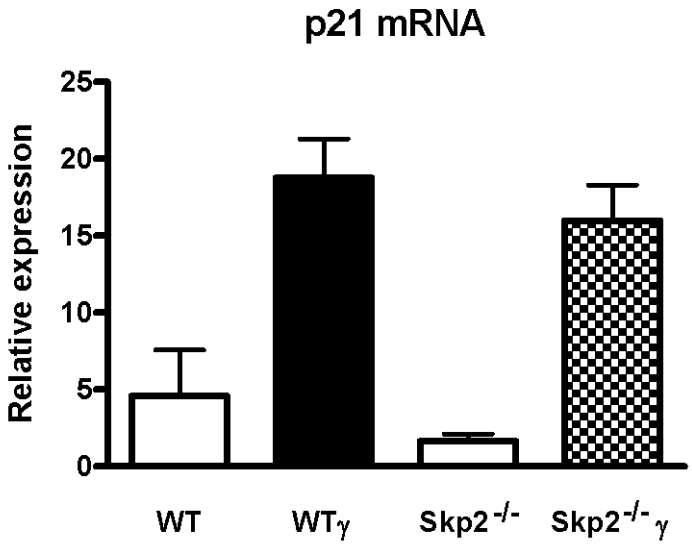

Figure 6. Real time RT-PCR analysis of p21 mRNA levels. Bar graph showing quantitative p21 mRNA analysis in wild-type (WT) and Skp2 $^{-/-}$ cerebella, without irradiation or three hours irradiated (γ).

The results (figure 6) show that, as expected, in the absence of irradiation p21 mRNA, levels are very low, presumably due to non activated p53. Upon irradiation, both in wild-type and in *Skp2* $^{-/-}$ cerebella, p21 mRNA levels increase to almost similar levels. This means p21 transcription is not significantly impaired after *Skp2* elimination.

2.1.1.2 – p21 expression is not decreased in the mitotic zone of *Skp2* $^{-/-}$ mice

To analyze whether the slight, albeit not significant, reduction of p21 mRNA in the *Skp2* $^{-/-}$ cerebella is reflected by reduced transcription within the mitotic zone, in situ hybridization was performed.

The in situ hibridization data (figure 7) confirmed the real time RT-PCR results. While only background signal can be detected in untreated mice, following irradiation p21 is induced

in the EGL of wild-type cerebella. A similar induction can be observed in *Skp2* $^{-/-}$ mice as well.

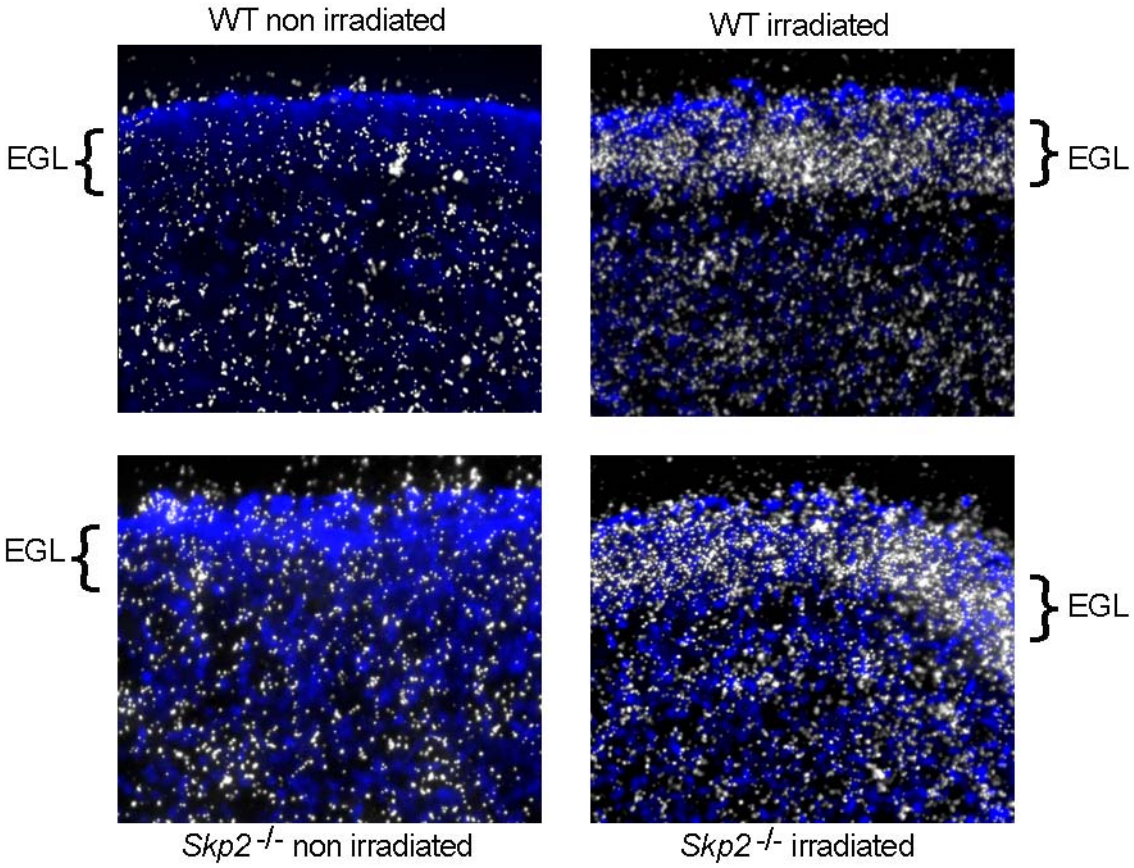

Figure 7. In situ hybridzation for p21 mRNA. Cerebellar sections from wild-type (WT) and Skp2 $^{-/-}$ *mice, either untreated or irradiated, were probed with radioactive antisense mRNA of p21 (white dots in darkfield microscopy); DAPI (blue) was used to counterstain nuclei. The position of the external granular layer (EGL) is indicated.*

Both the quantitative analyses and the histological examination by ISH demonstrate that irradiation-induced p21 mRNA transcription is not significantly impaired in the absence of Skp2.

2.1.2 – p27 transcript analysis

p27 can be repressed on the transcriptional level, in an irradiation-independent fashion, by the c-myc factor [Yang et al., 2001]. Therefore it can be speculated that, since both *p21* and *p27* are under the transcriptional regulation of c-myc, the lack of Skp2 may lead to an alteration in the activity of this transcription factor, causing at the same time downregulation of p21 and upregulation of p27.

Real time RT-PCR performed in parallel with p21 transcript analysis revealed a reduction of p27 mRNA, both in wild-type and *Skp2* $^{-/-}$ cerebella, after irradiation (figure 8). These data, also confirmed by ISH (figure 9), showed that Skp2 does not regulate p27 on the transcriptional level. Furthermore, ISH data demonstrated that p27 mRNA is present in the mitotic zone, supporting the view that Skp2 rather is involved in p27 proteasomal degradation in wild-type mitotic neuroblasts.

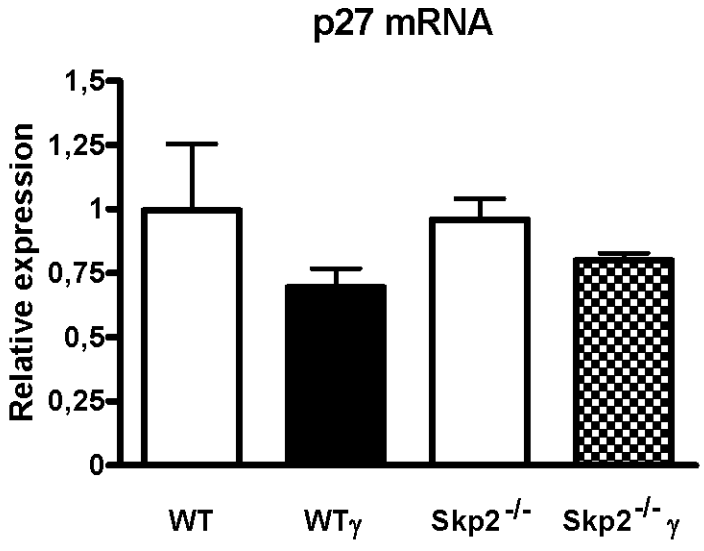

Figure 8. Real time RT-PCR analysis of p27 mRNA levels. Bar graph showing quantitative p27 mRNA analysis in wild-type (WT) and Skp2 $^{-/-}$ *cerebella, without irradiation or three hours irradiated (γ).*

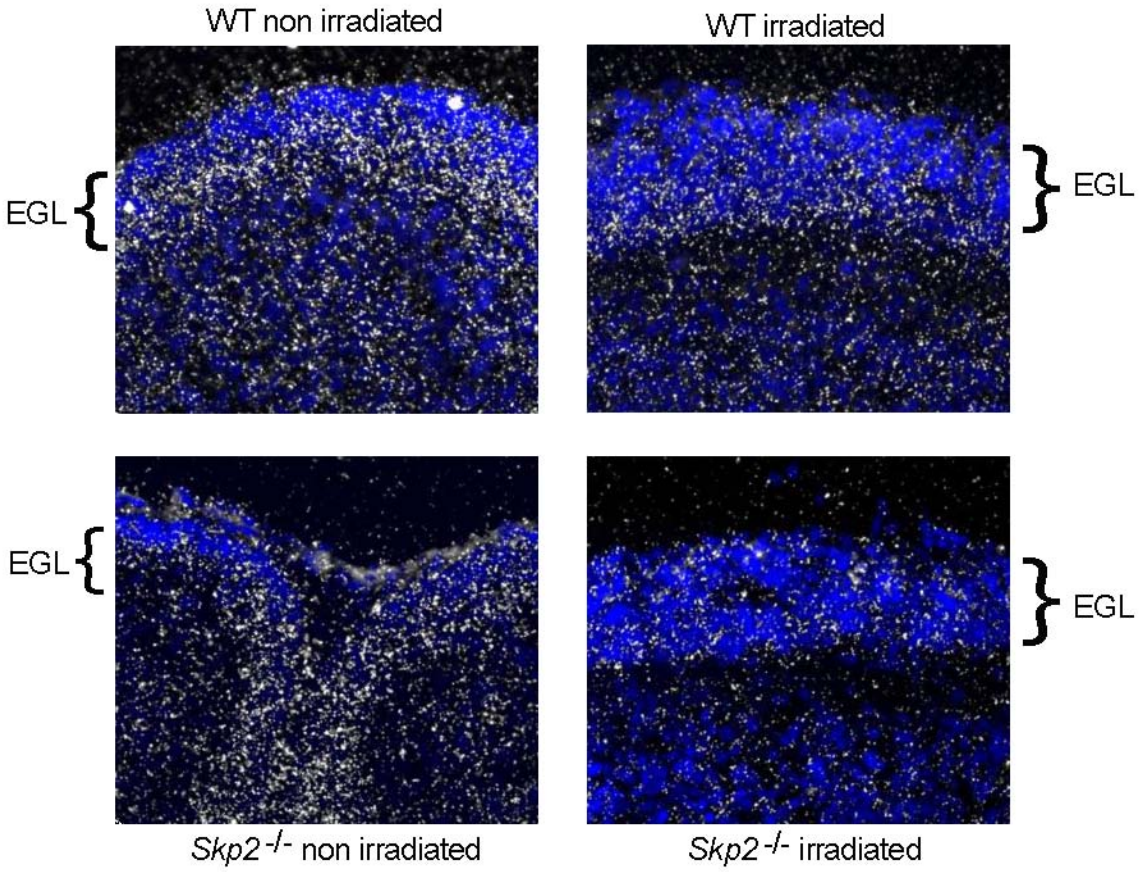

Figure 9. In situ hybridzation for p27 mRNA. Cerebellar sections from wild-type (WT) and Skp2 $^{-/-}$ mice, either untreated or irradiated, were probed with radioactive antisense mRNA of p27 (white dots in darkfield microscopy); DAPI (blue) was used to counterstain nuclei. The position of the external granular layer (EGL) is indicated.

2.2 – Elimination of *Skp2* and *p27* leads to ectopic p21 stabilization

As shown above, elimination of *Skp2* leads to accumulation of p27 in the cerebellum and in particular to ectopic localization of p27 also in the mitotic zone, where it is absent in wild-types (figure 7). This excess of p27 could therefore alter the formation of Cdk-CKI complexes in a stoichiometric manner, shifting the majority of these aggregates from p21-Cdk to p27-Cdk, since p21 and p27 share the same Cdk binding function [Cheng et al., 1999]. This in turn can lead to an increased presence of free p21, that can subsequently bind the 20S proteasome and be degraded [Touitou et al., 2001].

To investigate the role of p27 in the Skp2-p21 relationship, the *Skp2* $^{-/-}$ *; p27* $^{-/-}$ mice were employed. Although this mouse has been previously generated and characterized [Nakayama et al., 2004], using different breeding strategies and different genetic backgrounds, live animals were obtained extremely rarely (2 pups and one adult over 185 animals).

At P5 p21 cannot be found in the wild-type cerebellum in the absence of irradiation [Herzog et al., 2002]. Instead, in the *Skp2* $^{-/-}$ *; p27* $^{-/-}$ mouse, p21 can be detected in the absence of irradiation. To localize p21, a doublestaining using the anti-Zic1 antibody was carried out. Zic1 is increased during the maturation of neuroblasts, creating a gradient that peaks in the postmitotic zone. Since p21 was found at the interface between the mitotic and postmitotic zones (figure 10), it may be localized ectopically.

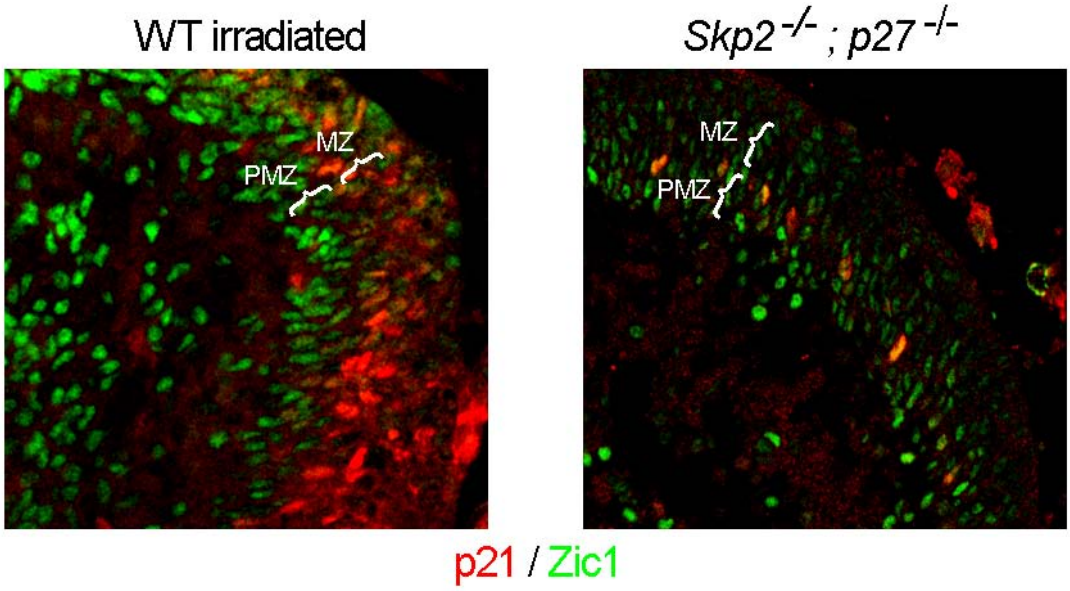

Figure 10. p21 localization in the wild-type irradiated (WTγ) and Skp2 $^{-/-}$ *; p27* $^{-/-}$ *non-irradiated cerebellum. Sections were immunostained for p21 (red) and Zic1 (green). The mitotic zone (MZ) and postimitotic zone (PMZ) are indicated.*

This result supports the concept of a competition between p21 and p27, since p21 stabilization is increased in the absence of p27. However, due to the limited numbers of

double knockouts, it was not possible to analyze also irradiated *Skp2* $^{-/-}$; *p27* $^{-/-}$ mice. Hence it is not clear whether p27 prevents p21 stabilization after DNA damage in *Skp2* $^{-/-}$ mice.

3 – Proteasomal degradation of p21

So far it has been shown that the lack of *Skp2* does not influence p21 mRNA levels and that there is no endoreplication in *Skp2* $^{-/-}$ cerebellar granular cells. In addition to transcriptional regulation, p21 is known to be regulated also by post-translational mechanisms, predominantly by the proteasome. Therefore it was investigated if the absence of *Skp2* led to a deregulation of these processes.

3.1 – Slice cultures

To address proteasomal degradation of p21, the cerebellar slice culture system was used. This approach allows to perform experiments in a tissue that retains the structural organization and presumably endogenous signaling mechanisms. The advantage of this experiment is the possibility to administer substances in a controlled manner, that are either not tolerated or not efficient in the animal. For instance, to block the proteasome in this experimental setting, Lactacystin was used. This assay was previously optimized and adapted regarding p21 regulation and it was found that the best results are obtained by explanting the tissue 1 hour after irradiation and culturing it for 2 hours in the absence or presence of the drug. Slices were analyzed by both Western blotting and immunofluorescence.

3.1.1 – Lactacystin administration increases p21 levels in slices from wild-type and *Skp2* $^{-/-}$ cerebella

The western blot analysis (figure 11) shows an increase of p21 in Lactacystin treated slices, both of wild-types and *Skp2* knockouts, supporting that p21 is degraded by the proteasome also in the absence of Skp2.

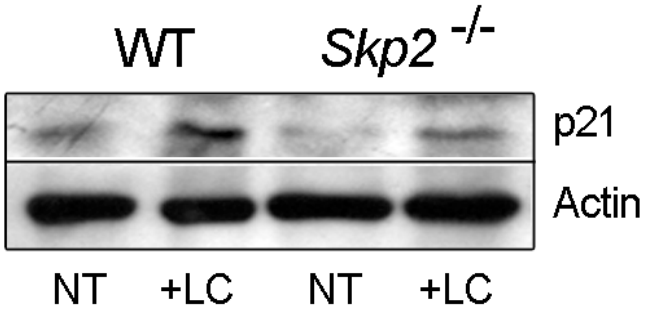

Figure 11. Proteasome blockage increases p21 levels in wild-type (WT) and Skp2 $^{-/-}$ *cerebellar slices. p21 levels were assayed by Western blot analysis on extracts of Lactacystin treated (+LC) and untreated (NT) cerebellar slices, obtained from one hour irradiated mice. Actin is shown as a loading control.*

Immunofluorescence done on slice sections (figure 12) confirms the low levels of p21 in the *Skp2* $^{-/-}$ non treated, while after Lactacystin treatment there is no obvious difference between wild-types and knockouts.

3.1.2 – p21 is degraded by the proteasome during S phase

BrdU administration to slices allows to identify cells in S phase. When the proteasome was blocked, p21 colocalized with BrdU, both in the wild-type and *Skp2* $^{-/-}$ (figure 12). This result means that, both in wild-type and *Skp2* $^{-/-}$ slices, p21 is degraded by the proteasome during S phase. Moreover, the increase in the number of p21-positive – BrdU-negative

cells in *Skp2* $^{-/-}$ slices supports that, in the absence of *Skp2,* p21 is degraded by the proteasome also in other cell cycle phases, presumably during G1 phase.

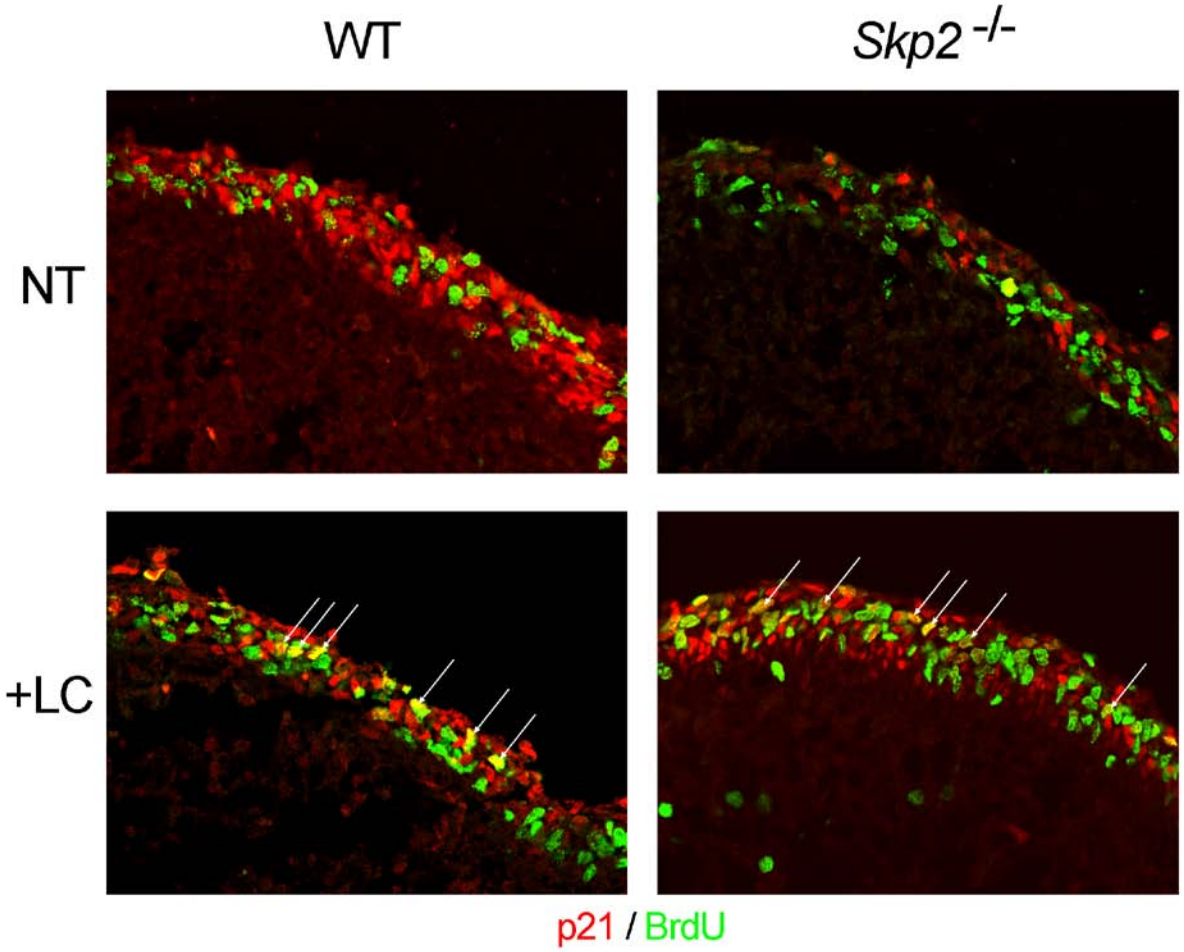

Figure 12. Proteasome blockage leads to increased p21 stabilization in wild-type (WT) and Skp2 $^{-/-}$ *cerebellar slices. Double immunostaining for p21 (red) and BrdU (green) performed on Lactacystin treated (+LC) and untreated (NT) cerebellar slices, obtained from one hour irradiated mice. Arrows indicate p21/BrdU double-positive cells (yellow cells).*

3.2 – *In vivo* proteasome inhibition by Bortezomib increases p21 levels in wild-type and *Skp2* $^{-/-}$ cerebella

Since cerebellar slices are only a close approximation, p21 degradation was addressed also *in vivo*, by Bortezomib application. Bortezomib is a well tolerated drug that inhibits the

activity of the proteasome and has been in clinical use for several years [reviewed in Einsele, 2010]. Bortezomib was administered to wild-type and *Skp2* $^{-/-}$ mice in a single 0,1 mg/kg dose by dorsal subcutaneous injection, 1 hour before irradiation. 3 hours after irradiation tissues were prepared for histology.

In vivo analyses of cerebella from wild-type and *Skp2* $^{-/-}$ mice confirmed the results from slice cultures: p21 degradation was prevented by inhibiting the proteasome (figure 13). In particular after proteasome blockage p21 was stabilized in S phase cells. Moreover the presence of many BrdU-negative p21-positive neuroblasts in the Bortezomib treated *Skp2* $^{-/-}$ suggests that p21 was also maintained in G1 phase cells.

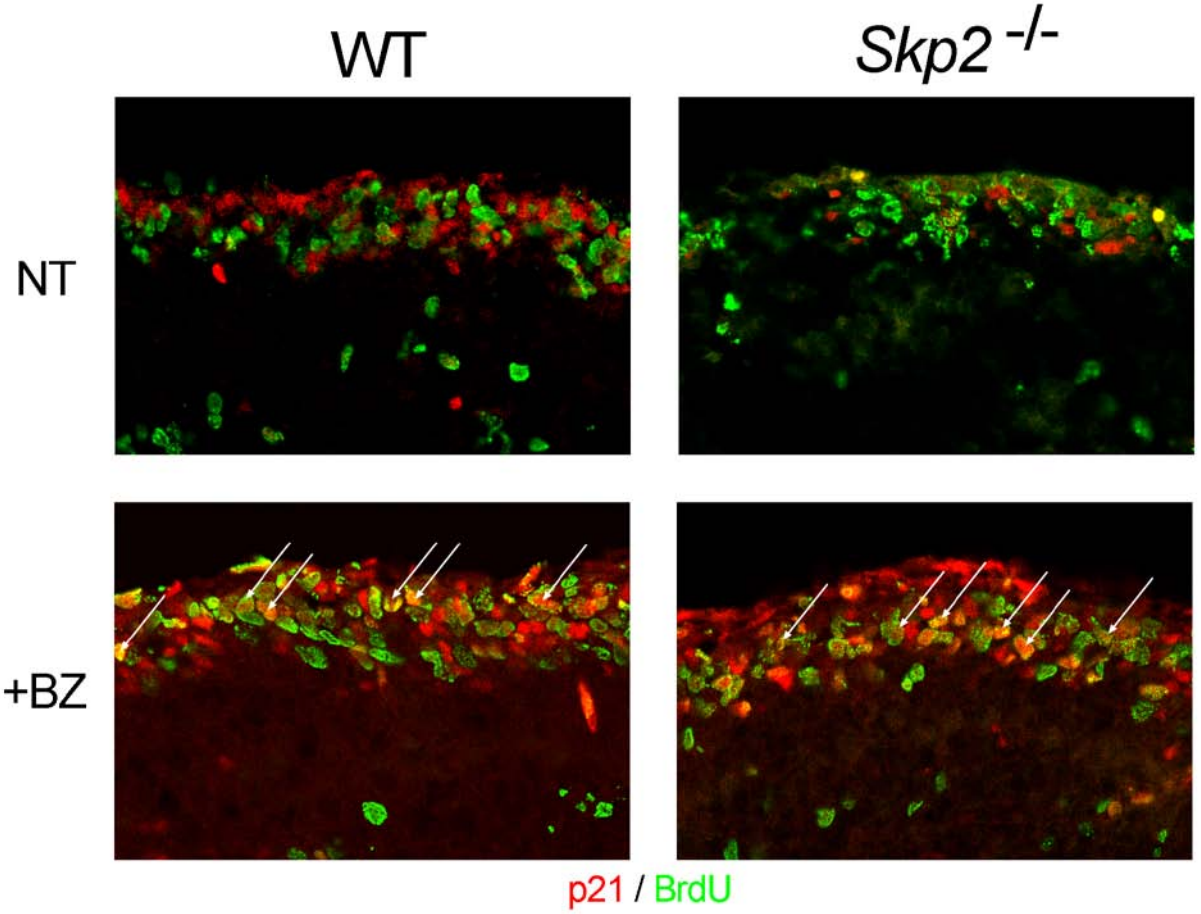

Figure 13. Proteasome blockage leads to increased p21 stabilization in wild-type (WT) and Skp2 $^{-/-}$ *cerebella. Double immunostaining for p21 (red) and BrdU (green) performed on cerebellar sections from three hours irradiated mice. Mice were either treated with*

Bortezomib (+BZ) or not treated (NT). Arrows indicate p21/BrdU double-positive cells (yellow cells).

4 – Skp2 independent proteasomal degradation of p21

The foregoing results demonstrated that Skp2 does not promote p21 degradation directly. In contrast, elimination of *Skp2* leads to more pronounced degradation of p21, presumably in G1 phase cells. The p53 regulators Mdm2 and Mdm4 have been shown to promote the degradation of p21 [Jin et al., 2003 ; Jin et al., 2008]. Therefore it was investigated whether these proteins are involved in p21 regulation in the absence of *Skp2*, in the developing cerebellum and retina.

4.1 – Functional analysis of the Mdm2/Mdm4 system regarding p21

For a functional analysis regarding the effect of Mdm2 and Mdm4 on p21 degradation *in vivo*, the drug Nutlin-3 was applied to P5 mice. Nutlin-3 binds to the C-terminus of both Mdm2 and Mdm4; upon Nutlin-3 binding, both Mdm2 and Mdm4 are no more able to interact with p53 [Laurie et al., 2006] and presumably also p21 binding is prevented [Enge et al., 2009]. Nutlin-3 was administered to mice, 1 hour before irradiation, by subcutaneous dorsal infusion in a dose of 20mg/kg. Three hours after irradiation, tissues were taken for biochemical analyses or mice were perfused for histological examination.

4.1.1 – Nutlin-3 treatment restores p21 in the cerebellum of *Skp2* $^{-/-}$ mice

Western blot analysis of irradiated cerebella treated with Nutlin-3 (figure 14) shows that this drug can upregulate the levels of p21 in the *Skp2* $^{-/-}$ mouse. This result supports that Mdm2 and/or Mdm4 are responsible for p21 degradation in the absence of *Skp2*.

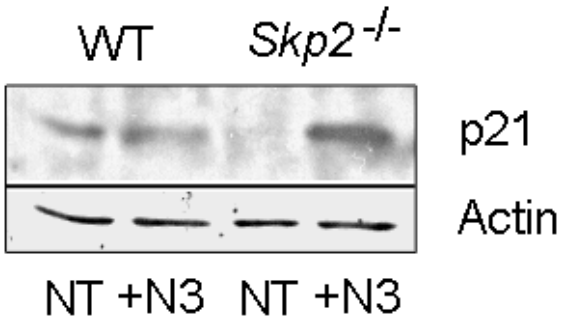

Figure 14. p21 levels are increased in Skp2 $^{-/-}$ cerebella after Nutlin-3 treatment. Western blot analysis of p21 levels in three hours irradiated wild-type (WT) and Skp2 $^{-/-}$ cerebella. Tissues were either Nutlin-3 treated (+N3) or non treated (NT). Actin is shown as a loading control.

4.1.2 – Nutlin-3 treatment restores p21 in *Skp2* $^{-/-}$ mitotic neuroblasts

To confirm that indeed p21 is degraded by Mdm2 and/or Mdm4 in mitotic neuroblasts, Nutlin-3 treated wild-type and *Skp2* $^{-/-}$ mice were examined histologically. A double immunofluorescence staining using antibodies against p21 and BrdU was carried out on cerebellar sections (figure 15).

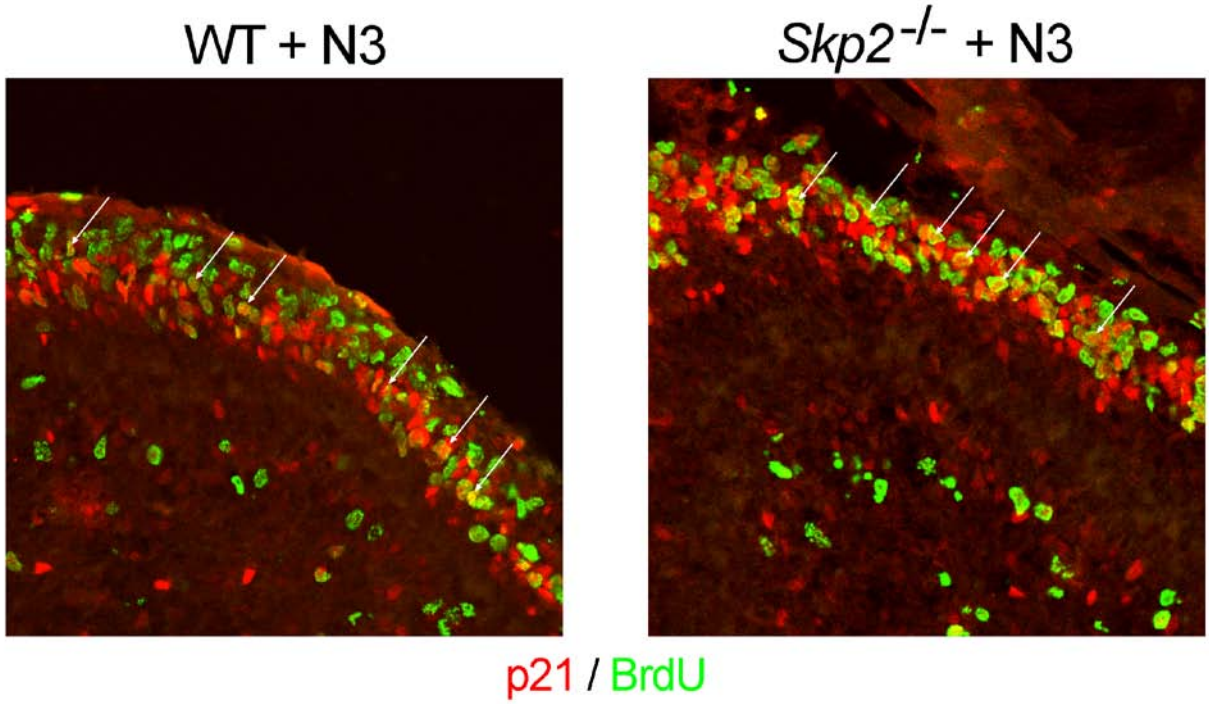

Figure 15. Nutlin-3 treatment leads to increased p21 stabilization in wild-type (WT) and

Skp2 $^{-/-}$ cerebella. Double immunostaining for p21 (red) and BrdU (green) performed on cerebellar sections from wild-type and Skp2 $^{-/-}$ irradiated and Nutlin-3 treated mice. Arrows indicate p21/BrdU double-positive cells (yellow cells).

Inhibition of Mdm2/Mdm4 by Nutlin-3 leads to stabilization of p21 in S phase cells, both in the wild-type and in the *Skp2 $^{-/-}$* animal. Moreover, in *Skp2 $^{-/-}$* cerebella, immunofluorescence demonstrates that a widespread population of cells becomes p21-positive after Nutlin-3 treatment, similar as in wild-types, suggesting that this drug also prevents p21 proteasomal degradation in G1 cells of the *Skp2 $^{-/-}$* mouse.

4.1.3 – Nutlin-3 treatment leads to p21 stabilization in G1 and S phase cells in the retina

To further support that p21 is degraded by Mdm2 and/or Mdm4 in G1 phase cells, the developing retina was used. Like for the cerebellum, the layering of the P5 retina is very precise and cells of particular cell cycle and differentiation stages can be found in discrete layers. In the mature retina, starting from the outermost, the layers are: the photoreceptor layer, the outer nuclear layer, the outer plexiform layer, the inner nuclear layer (INL), the inner plexiform layer and the ganglion cell layer [Purves, 2001].

Analyzing a cross-section of the P5 retina, that looks like a crescent with a central part and two extremities, cells in different maturation stages can be found in the inner nuclear layer in the central part or in the marginal parts of the section. While the marginal parts of the P5 retina harbor BrdU-positive cells, that are in S phase, the central part contains cells that are p27-positive and are in early G1 phase [Dyer and Cepko, 2001].

p21 was previously demonstrated [Herzog et al., 2002] to be transcribed in the inner nuclear layer of the P5 mouse retina as a consequence of p53 activation; subsequently, p21 protein is not stabilized under normal conditions. In the retina there is the opportunity of identifying the cell cycle phase of cells in the inner nuclear layer, either with BrdU (for S

phase cells) or with Chx10 (for G1 phase cells), which was not possible in the cerebellum. In fact, Chx10 is a transcription factor present in retinal progenitor cells of the inner nuclear layer that can express p27 [Green et al., 2003] and thus are in G1 phase.

In an effort to demonstrate p21 stabilization in G1 and S phase cells, the retina from Nutlin-3 treated animals was analyzed by double immunofluorescence, against p21/BrdU (figure 16) or p21/Chx10 (figure 17).

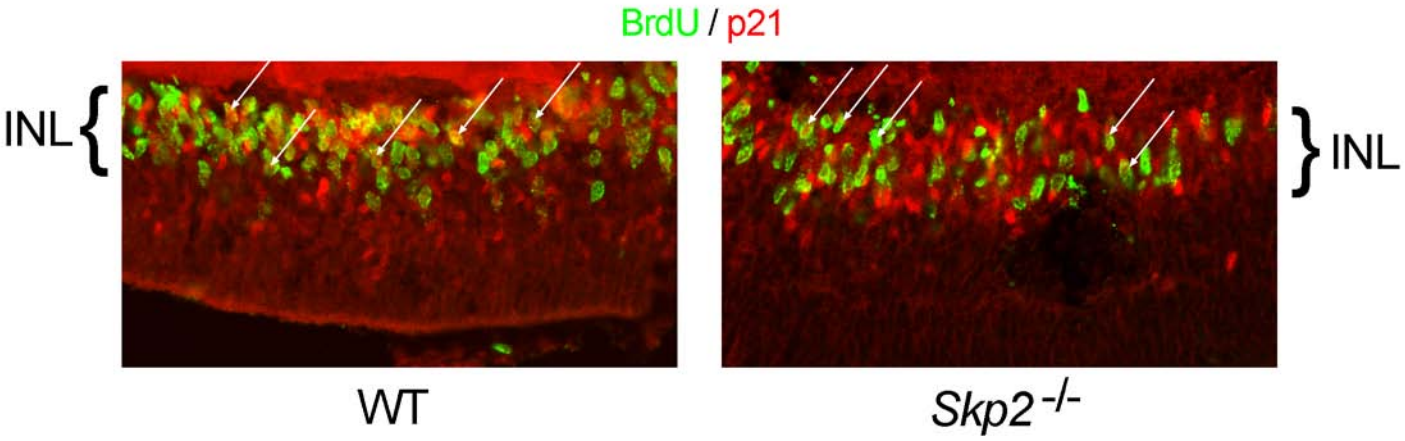

Figure 16. Nutlin-3 treatment leads to p21 stabilization in S phase cells, in the marginal part of the inner nuclear layer (INL) of wild-type (WT) and Skp2 $^{-/-}$ retinae. Double immunostaining for p21 (red) and BrdU (green) performed on retinal sections from Nutlin-3 treated mice. Arrows indicate p21/BrdU double-positive cells (yellow cells).

Like previously shown in the cerebellum, also in the retina p21/BrdU double-positive cells can be found after Nutlin-3 treatment, further supporting the notion that the *Mdm* system is responsible for p21 degradation during S phase.

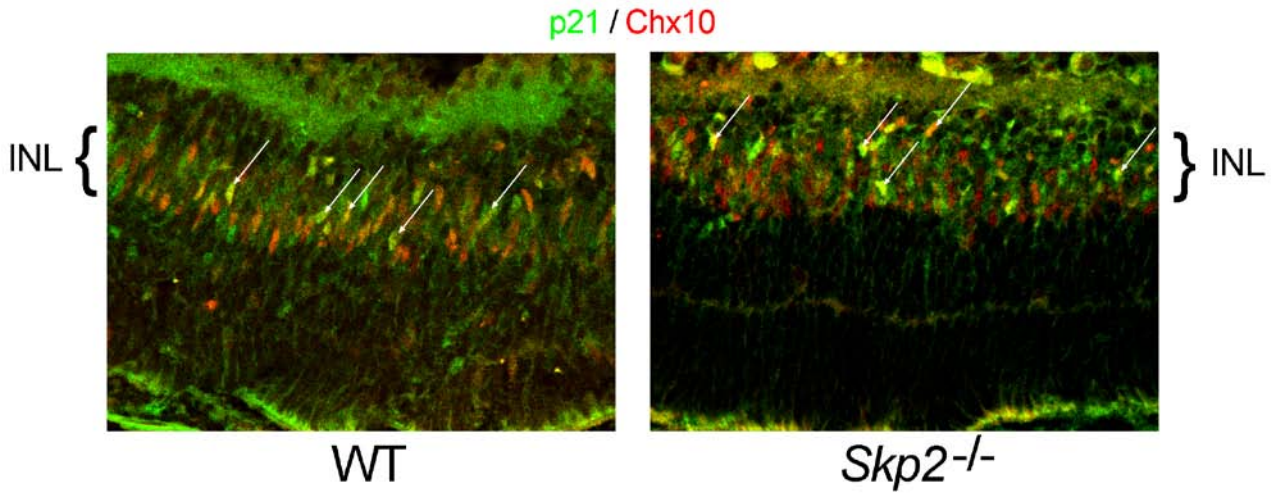

Figure 17. Nutlin-3 treatment leads to p21 stabilization in G1 phase cells, in the central part of the inner nuclear layer (INL) of wild-type (WT) and Skp2 $^{-/-}$ retinae. Double immunostaining for p21 (green) and Chx10 (red) performed on retinal sections from Nutlin-3 treated mice. Arrows indicate p21/Chx10 double-positive cells (yellow cells).

Furthermore, p21/Chx10 immunostaining of Nutlin-3 treated retinae demonstrated many double-positive cells. Therefore Mdm2 and/or Mdm4 are responsible for p21 degradation during G1 phase.

4.2 – Skp2 and Mdm2/Mdm4

The foregoing experiments demonstrated that pharmacological blockage of Mdm2/Mdm4 prevents p21 degradation and that the Mdm2/Mdm4 system is more active when *Skp2* was eliminated. Therefore, it was investigated whether this functional change is mediated by a quantitative alteration of either Mdm2 or Mdm4 levels.

4.2.1 – Analysis of Mdm2 levels regulation in the cerebellum

Mdm2 is characterized to be induced by p53 [Wu et al., 1993] and p53 is induced in the cerebellum after irradiation [Wood and Youle, 1995]. To assess whether Mdm2 is regulated in the cerebellum, a time-course Western blot analysis was carried out,

comparing wild-type cerebella taken at different time points after irradiation. Moreover *Skp2* $^{-/-}$ cerebella were analyzed, either non-irradiated or 3 hours after irradiation.

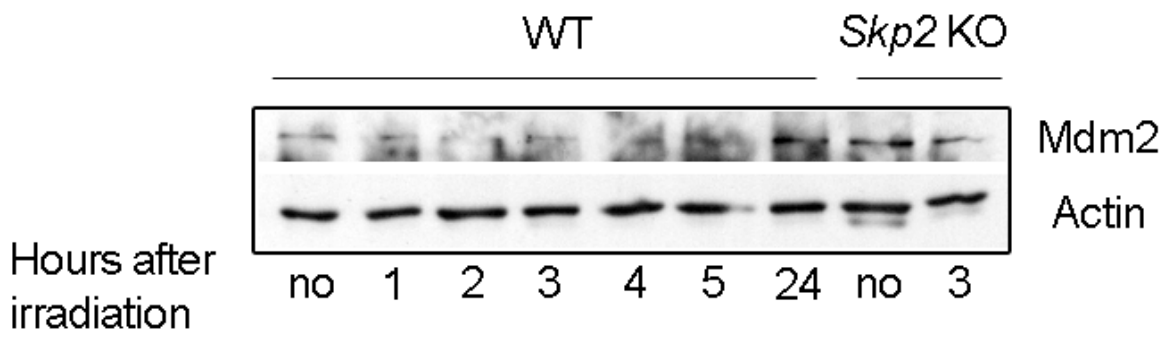

Figure 18. Time-course Western blot analysis of Mdm2 levels. Comparison is made between wild-type (wild-type) cerebella at different time points after irradiation and Skp2 $^{-/-}$ *(KO) cerebella, 3 hours after irradiation or non-irradiated. Actin is used as loading control.*

These results (figure 18) show that in wild-type mice a slight reduction of Mdm2 appears at 2-3 hours and the protein levels are higher at 24 hours, but overall the levels of Mdm2 are not dramatically altered. Regarding the *Skp2* $^{-/-}$ cerebella, Mdm2 is often found to be increased, although this was not confirmed in every animal (around 50% animals).

4.2.2 – Skp2 elimination does not alter Mdm2 transcription

To analyze whether the subtle difference in Mdm2 protein levels seen between wild-type and knockout (previous chapter) occurred at the transcriptional level, a quantitative transcript analysis was performed also on *Mdm2*. As described previously (chapter 2.1.1), Skp2 can influence p53-mediated transcription, for instance by binding to p300/CBP [Kitagawa et al., 2008].

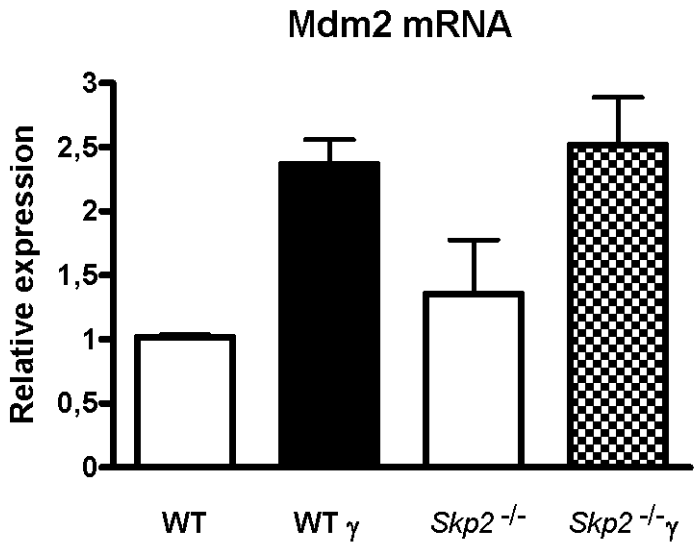

Figure 19. Real time RT-PCR analysis of Mdm2 mRNA levels. The bar graph shows values regarding the cerebella of wild-type (WT) and Skp2 $^{-/-}$ mice, either non-irradiated or three hours after irradiation (γ).

This result (figure 19) shows that, upon irradiation, Mdm2 mRNA is upregulated both in wild-type and *Skp2* $^{-/-}$ cerebella with no significant difference between wild-type and knockout, thus excluding any transcriptional influence of Skp2 on *Mdm2*.

4.2.3 – Mdm4 levels are not regulated either by irradiation or Skp2

Although Mdm4 is not known to be regulated by p53, it is subject to crossregulation with Mdm2 and other proteins regulated by irradiation [LeBron et al., 2006]. Therefore, the same time-course analyses for Mdm2 were also carried out with Mdm4 (figure 20). Mdm4 appears in Western blot analysis as multiple bands, at least four, that probably derive from alternative splicing or post-translational modifications. Indeed, these mechanisms of Mdm4 regulation have been described to occur in human cell lines and tissues [Mancini et al., 2009 ; Gentiletti et al., 2002], though they are not yet described in mouse.

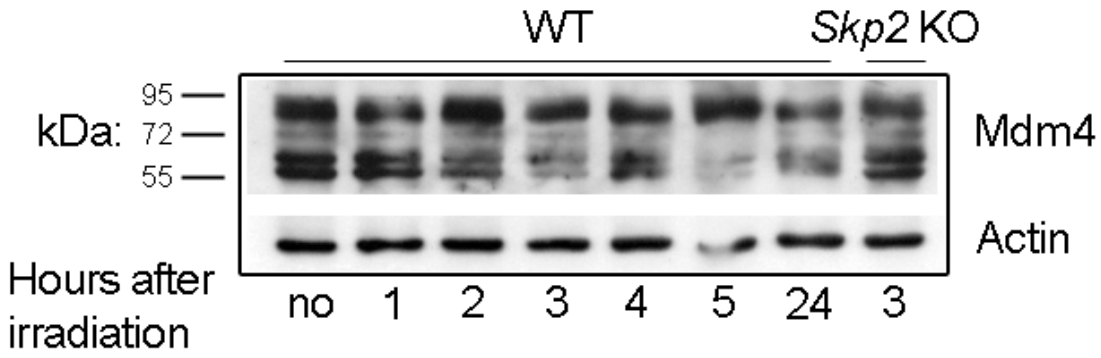

Figure 20. Time-course Western blot analysis of Mdm4 levels. Comparison is made between wild-type (wild-type) cerebella at different time points after irradiation and Skp2 $^{-/-}$ cerebella 3 hours after irradiation. Actin is used as loading control.

As shown in this time-course Western blot, the major protein band (around 90 kDa) relative to Mdm4 does not seem to be regulated in the cerebellum, either by irradiation or *Skp2* elimination.

5 – Viability of Skp2 $^{-/-}$ and Skp2 $^{-/-}$; p27 $^{-/-}$ mice

Analyzing the offspring of *Skp2* $^{+/-}$ parents, the normal mendelian ratio would lead to 25% *Skp2* $^{+/+}$, 50% *Skp2* $^{+/-}$ and 25% *Skp2* $^{-/-}$ pups. Instead an unbalance was evident already at postnatal day 3. While wild-types and heterozygotes display a 1:2 ratio, *Skp2* $^{-/-}$ mice are only 10% of the total offspring (figure 21, right). Instead analysis of E13.5 embryos revealed almost a normal mendelian ratio between all genotypes of the offspring (figure 21, left), implying that death of *Skp2* $^{-/-}$ mice occurs between E13.5 and P3.

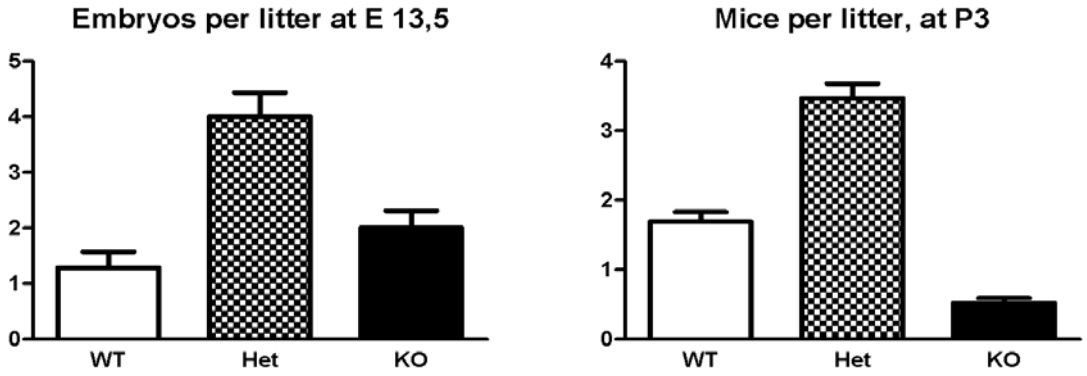

Figure 21. Comparison between the numbers of embryos and mice obtained by breeding Skp2 $^{+/-}$ *parents. The bar graph shows mice number for each genotype; wild-type: Skp2* $^{+/+}$*, Het: Skp2* $^{+/-}$ *, KO: Skp2* $^{-/-}$*.*

To obtain *Skp2* $^{-/-}$ *; p27* $^{-/-}$ mice, either *Skp2* $^{+/-}$ *; p27* $^{+/-}$ (expected ratio 1/16) or *Skp2* $^{-/-}$ *; p27* $^{+/-}$ parents (expected ratio 1/4) were intercrossed. With both strategies, out of 185 animals, 3 *Skp2* $^{-/-}$ *; p27* $^{-/-}$ mice were obtained (table 1).

Total animals: 185		Mice Number	
Genotype		Obtained	Expected
$Skp2^{+/+}$; $p27^{+/+}$ $Skp2^{+/+}$; $p27^{+/-}$ $Skp2^{+/+}$; $p27^{-/-}$ $Skp2^{+/-}$; $p27^{+/+}$ $Skp2^{+/-}$; $p27^{+/-}$ $Skp2^{+/-}$; $p27^{-/-}$	Skp2 +	152 (defined as 75%)	
	$Skp2^{-/-}$; $p27^{+/+}$	8 (3,9%)	12,7 (6,25%)
	$Skp2^{-/-}$; $p27^{+/-}$	22 (10,9%)	25,3 (12,5%)
	$Skp2^{-/-}$; $p27^{-/-}$	3 (1,5%)	12,7 (6,25%)

Table 1. Analysis of the effects of Skp2 and p27 elimination on mice viability. For each genotype the numbers of obtained and expected animals are shown. Relative percentages are shown in brackets.

By analyzing the numbers of obtained and expected animals for each *Skp2* and *p27* genotype, it was also possible to verify the effect of *p27* elimination regarding the *Skp2* $^{-/-}$ associated lethality. It was previously shown that viability is not altered when one or two alleles of *p27* are lacking [Fero et al., 1996]. Elimination of one allele of *Skp2* in combination with elimination of one or two alleles of *p27* also does not alter viability (data not shown). Thus, mice that have at least one *Skp2* allele were clustered in a single

population for this analysis. When considered that all mice of this group survived, this would represent 75% of the total offspring.

As expected (see above), *Skp2* $^{-/-}$mice were reduced. Moreover, *Skp2* $^{-/-}$ *; p27* $^{-/-}$ were even more reduced in respect to the expected mendelian ratio. Surprisingly however, when only one allele of *p27* was removed, *Skp2* $^{-/-}$ mice reached almost the expected mendelian ratio. This result suggests that the lethal effect of Skp2 elimination is mediated by the accumulation of p27, but a certain amount of p27 is needed for the survival of Skp2 $^{-/-}$ mice.

Discussion

The role of the cell cycle inhibitor p21 regarding apoptosis so far is not entirely clear. Although several studies have demonstrated that p21 can be proapoptotic in particular systems, there is much more evidence that p21 under unperturbed conditions is rather antiapoptotic [reviewed in Gartel and Tyner, 2002]. Provided p21 plays a crucial role in mediating apoptotic resistance, it would be important to understand its regulation for targeted tumor therapies.

Apparently two convenient systems to study p21 regulation processes *in vivo* are the murine cerebellum and the retina at the fifth postnatal day (P5). These experimental systems are characterized by the presence of proliferating cells [Altman and Bayer, 1997 ; Purves, 2001], that respond to irradiation with p21 expression.
In wild-type animals it has been shown that, after irradiation, in the mitotic zone of the cerebellum p53 triggers a signaling cascade that leads to both pro- and anti- apoptotic responses [Herzog et al., 2002]. While stabilization of p21 was linked to cellular survival, when p21 was not stabilized it was associated with activation of the apoptotic system. In the post-mitotic zone of the cerebellum, instead, neuroblasts express p27 and do not proliferate anymore; cells after cell cycle exit do not respond to DNA damage anymore with p53 activation.

The discrimination between cells that survive irradiation or undergo apoptosis evidently depends on the stabilization or degradation of p21. This in turn depends on p21 post-translational regulation [reviewed in Jung et al., 2010] and several proteins have been characterized to promote the degradation of p21 by the proteasome. The principal ubiquitin ligase subunit that has been associated to p21 ubiquitin-dependent degradation is Skp2 [Bornstein et al., 2003], while Mdm2 and Mdm4 have been described to promote the ubiquitin-independent degradation of p21 [Jin et al., 2003 and 2008]. Recently it was

shown that Mdm2 induces a conformational change in p21 that enhances its binding to the proteasome [Xu et al., 2010].

However it is not clear which of the two mechanisms is crucially involved in p21 degradation processes. The ubiquitin-ligase Skp2 has been described to promote p21 poly-ubiquitination in *in vitro* ubiquitination assays, carried out using murine embryonic fibroblasts [Bornstein et al., 2003], and *Skp2* gene amplification has been shown to promote ubiquitination and degradation of p21 in hepatocellular carcinoma cell lines [Calvisi et al., 2009]. Instead, in other cellular systems, Skp2 has been shown to be dispensable for p21 degradation [Chen et al., 2004]. Indeed p21 is able to bind to the 20S proteasome directly [Touitou et al., 2001], leading to its degradation. For instance in HeLa cells, removal of *Skp2* leads to stabilization of p27, but not of p21 [Zhang et al., 2009]. Similarly in transformed fibroblasts, p21 degradation occurs in a Skp2-independent manner after UV irradiation [Stuart and Wang, 2009]. Like for Skp2, also Mdm2 and Mdm4 have been shown to promote p21 degradation in murine embryonic fibroblasts [Jin et al., 2003 and 2008], suggesting that these cellular models do not allow to discriminate which of the two p21 degradation mechanisms is actually used. It was therefore chosen to carry out an *in vivo* analysis, regarding the role of Skp2 and, subsequently, on the role of Mdm2/Mdm4 regarding p21 degradation.

Skp2 expression is associated with both physiological and aberrant cell proliferation [reviewed in Hershko, 2008]. Also in the mouse cerebellum at P5 the Skp2 protein is at its maximal expression [Herzog, personal communication]. To carry out a functional analysis for the role of Skp2 regarding p21 degradation, the *Skp2* knockout mouse [Nakayama et al., 2000] was employed. Provided that p21 was degraded by SCF containing Skp2, it would have been expected that the *Skp2* $^{-/-}$ mice stabilized more p21 than a wild-type. On the contrary, previous data showed that p21 is strongly reduced in the mitotic zone of

Skp2$^{-/-}$ mice [Bürkle and Herzog, unpublished]. Instead p27 was demonstrated to be increased in the *Skp2* $^{-/-}$ cerebellum. Like in wild-types, p27 is stabilized in the postmitotic zone; however, in addition, p27 can be found in the mitotic zone of *Skp2* knockouts. In fact, p27 has been shown to be a major target for Skp2-mediated degradation both *in vitro* and *in vivo* [reviewed in Nakayama and Nakayama, 2005].

To clarify the role of Skp2 in p21 regulation, a first investigation was carried out regarding the localization of stabilized p21 in the particular cell cycle phases.

To evaluate the impact of granular cells in different cell cycle stages, regarding the levels of p21, their relative amounts were considered (G1= 45% ; S= 42% ; G2= 10,5% ; M= 2,5% [Fujita, 1967]). It can therefore be speculated that M phase cells give no relevant contribution to the levels of p21 and also that G2 cells cannot be responsible for the dramatic difference in p21 amount between wild-type and *Skp2* $^{-/-}$. Instead cells in G1 and S phases represent the major populations in the P5 cerebellum, thus impaired p21 stabilization in either phase could lead to p21 diminution in the *Skp2* knockout mice.

In fact, investigation of M phase cells was not possible, since the histological identification of this population relies on the nuclear staining of phospho-histone H3, which after γ-irradiation is downregulated [Xu et al., 2002], and no M-phase specific interactor of p21 could be employed.

In G2 phase, p21 has been previously demonstrated to be stabilized in several experimental systems [reviewed in Taylor and Stark, 2001]. Also in the cerebellum *in vivo*, it was found that p21 can be stabilized in G2 phase both in wild-type and *Skp2* $^{-/-}$ animals; p21 was previously demonstrated [Herzog, personal communication] to colocalize with phospho-histone H3, suggesting a stabilization in G2 phase. Moreover, stabilization of p21 was confirmed by observing that p21 can bind to Cdk1 and cyclinA [Herzog, personal

communication], which are known interactors of p21 during G2 phase [reviewed in Taylor and Stark, 2001].

p21 was shown to bind Cdk4, Cdk6 and cyclinD1 [Herzog, personal communication]. However, an interaction of p21 with these cell cycle regulators does not necessarily support a stabilization of p21 during G1 phase. In fact it has been demonstrated that p21 can bind Cdk4, Cdk6 and cyclinD1 also during other cell cycle phases, in particular in G2 phase [Gabrielli et al., 1999]. Since previous data showed that p21 is not stabilized during S phase in the cerebellar granule cells [Herzog, personal communication], it is reasonable to assume that G1 phase neuroblasts are the main cell population responsible for p21 decrease in the absence of *Skp2.*

To identify the mechanism responsible for this reduction of p21 in G1 phase cells, several hypotheses were investigated.

Recent evidence [Espinosa and Luo, 2008] has shown that in the cerebellum exist two subpopulations of granular neuroblasts, that are in different maturation stages and are located either in the outer or in the inner part of the mitotic zone. Since in the *Skp2* $^{-/-}$ cerebella p21 is reduced predominantly in the outer part, one possibility is that in the absence of *Skp2* the relative proportion of both populations is altered and one can stabilize less p21. However, given the low amount of cells that could be affected, this alteration cannot be accounted for the diminution in p21 levels, as it was found in the *Skp2* $^{-/-}$ cerebella.

Assuming that p21 was downregulated during G1 phase, it was analyzed whether mitotic neuroblasts suffered from an impairment of the G1 cell cycle checkpoint; this would lead to reduced p53 activation and subsequently less p21 production. In fact endoreplication has been characterized in *Skp2* $^{-/-}$ hepatocytes and embryonic fibroblasts [Nakayama et al., 2000]. Therefore it was tested whether the two hallmarks of endoreplication, increase in nuclear size and centrosome amplification [reviewed in Edgar and Orr-Weaver, 2001],

were detectable in the cerebellum. The accumulation of genetic materal was previously investigated [Ulrich and Herzog, unpublished], showing no difference in nuclear DNA content between wild-type and *Skp2* $^{-/-}$ cerebella. Since that experiment was performed on the whole cerebellum, it was necessary to restrict the analysis to cells in the mitotic zone. Indeed, measurement of the nuclear size of the mitotic neuroblasts, as well as evaluation of centrosome amplification in granular cell cultures, confirmed that no endoreplication was ongoing in that cell population, either in wild-type or in *Skp2* $^{-/-}$ animals.

Although G1 phase, and presumably also the G1 checkpoint, was not perturbed in neuroblasts, it has been shown that in *Skp2* $^{-/-}$ mice higher p27 levels lead to reduced body size [Nakayama et al., 2004] and presumably slower cell cycle kinetics in mitotic granular cells. In fact, in the *Skp2* $^{-/-}$ cerebellum one evident phenotype is the increased presence of p27; in the absence of *Skp2*, p27 is stabilized not only in the postmitotic zone, like in the wild-type animals, but also in the mitotic zone.

It has been shown that p27 elimination leads to post-transcriptional upregulation of p21 [Kwon et al., 2002]; moreover, p21 and p27 can functionally substitute each other for binding CDK complexes [Cheng et al., 1999] and, depending on the cell type, *in vivo* elimination of p21 and p27 shows additive effects [Jirawatnotai et al., 2003 ; Holsberger et al., 2005]. Therefore, a potential interplay between these two inhibitors was analysed in more detail at both transcriptional and post-transcriptional levels.

In fact, Skp2 has been demonstrated to regulate the activity and halflife of the c-Myc transcription factor [Kim et al., 2003 ; von der Lehr et al., 2003]. Theoretically this transcription factor could link together Skp2, p21 and p27, since both p21 and p27 promoters contain elements that can either be activated or repressed by c-Myc and its binding partners [Seoane et al., 2002 ; Yang et al., 2001]. This notion raised the possibility for an inverse regulation, where the absence of *Skp2* could lead on one side to the

decrease of p21 mRNA and on the other side to a rise in p27 mRNA, by a change in c-Myc activity.

p21 mRNA was investigated by real-time RT-PCR and found, as expected, to have very low basal levels in the absence of irradiation, presumably due to the fact that the p53 system was not triggered. After irradiation, p21 mRNA was strongly induced, both in wild-type and *Skp2* $^{-/-}$ cerebella, with a slight, albeit non significant, decrease in the latter. To further support that no reduction occurred in the mitotic zone, in situ hybridization was carried out. This analysis showed that there was no difference between wild-type and *Skp2* $^{-/-}$ external granular layers, confirming that Skp2 had no transcriptional influence on p21. In addition p27 transcript analyses were carried out, demonstrating that the absence of *Skp2* did not have an influence on transcription and that irradiation leads to a significant decrease in p27 mRNA, by unknown mechanisms.

After evaluating transcriptional regulation, the post-translational interference between p21 and p27 proteins was investigated. Since p27 is able to bind the same cyclin-Cdk complexes that bind p21 [Cheng et al., 1999], one possibility is that a competition between the two inhibitors arises in the mitotic zone. In a wild-type animal only p21 is present, so it can bind cyclin-Cdk complexes and be stabilized; instead, in the *Skp2* $^{-/-}$ cerebellum the ectopic presence of p27 in the mitotic zone could be responsible for occupying cyclin-Cdk complexes, hence leaving p21 unbound. p21 could in turn bind the proteasome more efficiently, as free non ubiquitylated p21 protein has been shown to be rapidly degraded by the proteasome [Chen et al., 2004].

To investigate the influence of p27 on p21, *Skp2/p27* doubleknockout mice [Nakayama et al., 2004] were employed, but limited animal numbers did not allow the study to be carried out completely. In fact, using different breeding strategies and different genetic backgrounds, only three double knockouts were obtained and they were not subjected to γ-irradiation. Nevertheless, the analysis of non-irradiated *Skp2* $^{-/-}$; *p27* $^{-/-}$ mice showed that

p21 can be stabilized also in the absence of irradiation, suggesting that p27 can indeed have a role in destabilizing p21. Still, it is not clear whether this phenomenon can be applicable to the irradiation context. Indeed p21 in the *Skp2* $^{-/-}$; *p27* $^{-/-}$ might possess an ectopic localization, since it was found at the interface between mitotic and postmitotic zones. Moreover, it is important to note that many post-translational modifications that affect p21 stability occur after γ-irradiation and these were not activated in this context.

Apparently the lack of *p27* can lead to p21 stabilization in the absence of *Skp2*, whereas the lack of *Skp2* leads to a diminution of p21 after radiation.

Using the cerebellar slice culture system it was shown that p21 is degraded by the proteasome during S phase [Herzog, personal communication]. Similarly, this system was used to compare the levels of p21 in wild-type or *Skp2* $^{-/-}$ slices, with administration of the proteasome inhibitor Lactacystin. These experiments demonstrated that the levels of p21 in the untreated *Skp2* $^{-/-}$ slices were, similar like *in vivo*, much lower as compared to the wild-type, while after proteasome blockage the levels became almost equal. Proteasome blockage experiments were also performed by applying Bortezomib, that in contrast to Lactacystin can also be used *in vivo*. Bortezomib was therefore administered to wild-type and *Skp2* $^{-/-}$ mice and analyses showed consistent results. With these proteasome inhibition experiments it was possible to demonstrate that, regardless of Skp2 influence, the proteasome is responsible for p21 degradation. Moreover these results demonstrated that the lower p21 levels in *Skp2* $^{-/-}$ mice are also dependent on proteasomal degradation. In addition, the histological analyses carried out on both cerebellar slices and cerebellar sections demonstrated that S phase cells are able to stabilize p21 upon proteasome blockage, regardless of *Skp2* status. This histological examination also allowed to observe that after proteasome blockage a great number of BrdU-negative cells became p21-

positive in the *Skp2* $^{-/-}$ cerebellum, suggesting that p21 is degraded by the proteasome also in G1 phase cells.

Apparently Skp2 does not degrade p21. Rather Skp2 leads to stabilization of p21, as shown by proteasome inhibition experiments. p21 destabilization has been shown to be promoted by Mdm2 and Mdm4, as these two proteins were characterized to bind p21 and facilitate its ubiquitin-independent proteasomal degradation [Jin et al., 2003 and 2008].
So far a direct involvement of Skp2 in Mdm2/Mdm4 regulation has not been shown. To carry out a functional analysis regarding the role of the Mdm2/Mdm4 system in the degradation of p21, the drug Nutlin-3 was used. This compound is able to bind to the C-terminus of both Mdm2 and Mdm4, preventing their interaction with p53 [Laurie et al., 2006]. It was therefore hypothesized, based on investigations by Enge et al. (2009), that Nutlin-3 could also prevent the effect of Mdm2 and Mdm4 on p21. Although Nutlin-3 has been used for adult mice in a chronical oral administration, it can also be administered intraperitoneally [reviewed in Shangary and Wang, 2009]. It must be considered that the K_i of Nutlin-3 for Mdm2 is 0,7 µM and the K_i for Mdm4 is 28 µM [Laurie et al., 2006]. Theoretically these K_is correspond to concentrations of around 4 mg/l and 16mg/l. The dose chosen according to adult mice treatments [reviewed in Shangary and Wang, 2009] was 20mg/Kg administered subcutaneously, which, considering pharmacological interactions, supposedly allowed a complete inhibition of Mdm2 and a partial inhibition of Mdm4.
Nutlin-3 administration restored p21 levels in the *Skp2* $^{-/-}$ cerebellum, similar like in wild-types. Moreover, histological analyses showed that Nutlin-3 treatment can lead to p21 stabilization in S phase cells, that under normal conditions do not stabilize it. This result is consistent with the effect of proteasome blockage and thus implies that either Mdm2 and/or Mdm4 are responsible for p21 degradation during S-phase in normal neuroblasts.

In addition, similar to the result obtained with Bortezomib, a widespread population of BrdU-negative cells stabilized p21 in the *Skp2* $^{-/-}$ after Nutlin-3 treatment, suggesting that also G1 phase cells are involved in Mdm2/Mdm4-mediated proteasomal degradation of p21.

As mentioned above, identification of G1 phase cells by co-immunoprecipitation was not possible, since Cdk4 and cyclinD1 interact with p21 also during other cell cycle steps. Thus, to identify cells in G1 phase unambigously, the developing retina was used as an additional *in vivo* model. The central part of the inner nuclear layer contains at P5 cells that are in different cell cycle stages, including G1 [Cepko and Dyer, 2001] and these cells can be identified by Chx10 immunostaining [Green et al., 2003]. It was previously demonstrated that cells in the inner nuclear layer express p21 mRNA as a consequence of irradiation, but p21 protein is not subsequently stabilized [Herzog et al., 2002]. As predicted from the experiments carried out in the cerebellum, G1 phase cells in the inner nuclear layer of both wild-type and *Skp2* $^{-/-}$ mice could stabilize p21 after Nutlin-3 administration. This result directly proved that Mdm2 and/or Mdm4 are also able to promote p21 degradation during G1 phase, thereby suggesting that this is the main mechanism responsible for the lack of p21 in the cerebellum due to *Skp2* elimination.

To gain mechanistic insights regarding the degradation of p21 in the *Skp2* $^{-/-}$ cerebellum, a quantitative analysis of both Mdm2 and Mdm4 was carried out. It was found that Mdm2 protein levels are slightly regulated by irradiation, while Mdm4 is not. Regarding the influence of Skp2, no effect was evident on Mdm4, while Mdm2 protein, but not Mdm2 mRNA, appeared to be upregulated in the absence of *Skp2.* However, the upregulation of Mdm2 was not evident in every *Skp2* $^{-/-}$ animal; indeed, also p21 levels are not always homogeneous between *Skp2* $^{-/-}$ mice, as all of them display a reduction of p21 levels, but this effect can be more or less severe. This could be correlated with a major or minor

upregulation of Mdm2. In this context, it is important to note that regulation of the Mdm2/Mdm4 system occurs mostly by post-translational modifications that do not affect protein levels [Wade et al., 2010]. Therefore, although Mdm2/Mdm4 levels are not dramatically altered in the *Skp2* $^{-/-}$ mouse, the absence of Skp2-mediated post-translational modifications could rather lead to a change in the activity of Mdm2 and/or Mdm4, causing p21 destabilization.

In the effort to obtain *Skp2* $^{-/-}$; *p27* $^{-/-}$ mice, an analysis was carried out regarding lethality associated with the lack of *Skp2* and *p27* genes. On the contrary to what was previously observed [Nakayama et al., 2000], mice lacking *Skp2* were not normally viable, in fact at P3 *Skp2* $^{-/-}$ mice numbers were reduced in respect to the expected mendelian ratio. Since E13.5 embryos displayed an almost mandelian ratio, it can be concluded that death occurs within this time-frame, albeit the causes of death are unknown. *Skp2* $^{+/-}$; *p27* $^{+/-}$ or *Skp2* $^{-/-}$; *p27* $^{+/-}$ mice were crossbred to obtain *Skp2* $^{-/-}$; *p27* $^{-/-}$ mice and with this analysis it was possible to investigate the influence of p27 on rescuing the Skp2-dependent lethality. Nakayama et al. (2004) demonstrated that removing two alleles of *p27* prevents endoreplication and reduced body size in *Skp2* knockout animals. Surprisingly, when *p27* was completely removed from *Skp2* $^{-/-}$ mice, the number of obtained animals was even more reduced when compared to *Skp2* knockouts. Instead, when only one allele of *p27* was eliminated, *Skp2* $^{-/-}$; *p27* $^{+/-}$ mice displayed an almost normal ratio. This result implies that eliminating one allele of *p27* leads to a reduction in p27 protein levels that is sufficient to avoid the problems related to the absence of *Skp2*, but a certain amount of p27 is required for the survival of *Skp2* $^{-/-}$ mice.

Overall, this study clarified that, in the developing nervous system, *Skp2* elimination does not cause endoreplication, nor does it affect transcription of p21, p27 or Mdm2. Most importantly, pharamacological inhibition of the proteasome with Lactacystin and

Bortezomib proved that Skp2 does not promote p21 proteasomal degradation. Instead p21 is more stable in the presence of Skp2. Moreover, Nutlin-3 application allowed to identify in either Mdm2 and/or Mdm4 the molecule responsible for the decrease in p21 levels that follows *Skp2* elimination. This mechanism is post-translational, since proteasome blockage and Nutlin-3 application lead to a similar stabilization of p21.

It is therefore possible to speculate that Skp2 has a role between Mdm2/Mdm4 and p21. Mechanistic studies regarding the interaction between Skp2 and Mdm2/Mdm4 suggested that quantitative alterations presumably are not involved in the activity of Mdm2 and Mdm4 regarding p21 destabilization in *Skp2* $^{-/-}$ animals.

Further molecular analyses will be required to clarify the relationship between Skp2, Mdm2/Mdm4 and p21. In particular, silencing experiments on Mdm2 and Mdm4 should clarify their role regarding the proteasomal degradation of p21 in the absence of *Skp2*. In addition, it will be of interest whether Skp2 can directly interact with Mdm2/Mdm4 or if other molecules, like ARF, are involved.

This additional level of regulation could be exploited by pharmacological treatments, acting either on Mdm2/Mdm4 or on Skp2, thereby providing a possibility for the regulation of p21 stability and hence for the modulation of the apoptotic response to irradiation.

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