

# MDM2 and Fbw7 cooperate to induce p63 protein degradation following DNA damage and cell differentiation

Francesco Galli<sup>1,\*</sup>, Mariangela Rossi<sup>2,\*</sup>, Yuri D'Alessandra<sup>1</sup>, Marco De Simone<sup>1</sup>, Teresa Lopardo<sup>1</sup>, Ygal Haupt<sup>3</sup>, Osnat Alsheich-Bartok<sup>4</sup>, Shira Anzi<sup>5</sup>, Eitan Shaulian<sup>5</sup>, Viola Calabrò<sup>2</sup>, Girolama La Mantia<sup>2</sup> and Luisa Guerrini<sup>1,†</sup>

<sup>1</sup>Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

<sup>2</sup>Dipartimento di Biologia Strutturale e Funzionale, Università di Napoli Federico II, 80126 Italy

<sup>3</sup>Research Division, The Peter MacCallum Cancer Centre, St. Andrew's Place, East Melbourne 3002, Victoria, Australia

<sup>4</sup>Lautenberg Center for General and Tumor Immunology, Lautenberg Center for general and Tumor Immunology, The Hebrew University, Hadassah Medical School, Jerusalem 91120, Israel

<sup>5</sup>Department of Biochemistry and Molecular Biology, The Institute for Medical Research–Israel-Canada, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

\*These authors contributed equally to this work

†Author for correspondence ([luisa.guerrini@unimi.it](mailto:luisa.guerrini@unimi.it))

Accepted 15 April 2010

*Journal of Cell Science* 123, 2423–2433

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doi:10.1242/jcs.061010

## Summary

Tight control of p63 protein levels must be achieved under differentiation or apoptotic conditions. Here, we describe a new regulatory pathway for the  $\Delta Np63\alpha$  protein. We found that MDM2 binds  $\Delta Np63\alpha$  in the nucleus promoting its translocation to the cytoplasm. The MDM2 nuclear localization signal is required for  $\Delta Np63\alpha$  nuclear export and subsequent degradation, whereas the MDM2 ring-finger domain is dispensable. Once exported to the cytoplasm by MDM2, p63 is targeted for degradation by the Fbw7 E3-ubiquitin ligase. Efficient degradation of  $\Delta Np63\alpha$  by Fbw7 (also known as FBXW7) requires GSK3 kinase activity. By deletion and point mutations analysis we have identified a phosphodegron located in the  $\alpha$  and  $\beta$  tail of p63 that is required for degradation. Furthermore, we show that MDM2 or Fbw7 depletion inhibits degradation of endogenous  $\Delta Np63\alpha$  in cells exposed to UV irradiation, adriamycin and upon keratinocyte differentiation. Our findings suggest that following DNA damage and cellular differentiation MDM2 and Fbw7 can cooperate to regulate the levels of the pro-proliferative  $\Delta Np63\alpha$  protein.

**Key words:** DNA damage, Fbw7, MDM2, p63

## Introduction

Loss of the p53 tumor suppressor protein has been shown to play an important role in promoting tumor growth, whereas its activation following a variety of stress signals promotes a potent cell growth inhibition (Kruse and Gu, 2009; Vousden and Lane, 2007). p63, a p53-related protein, has been shown to activate p53-responsive genes and induce apoptosis in certain cell types, however the biological role of p63 appears to be more complex than that of a classic tumor suppressor (Flores et al., 2005). A clear role for p63 during embryonic development has been established by the study of p63 knockout mice. These mice have severe defects in epithelial stratification and fail to form epidermal appendages such as teeth, hair and glands (Mills et al., 1999; Yang et al., 1999). Also, it is debated whether p63 might act as a tumor suppressor or an oncogene. p63 is not mutated in tumors, which is in stark contrast to the high mutation status of p53 in a large group of cancer types. Conversely, p63 is often overexpressed and amplified in cancer, thus suggesting that p63 provides cancer cells with a selective advantage (Thurfjell et al., 2004; Finlan et al., 2007).

The p63 gene is expressed as multiple isoforms because of alternative transcription start sites and splicing at the C-terminus (van Bokhoven and Brunner, 2002). The six major isoforms of p63 have several conserved regions common to the p53 family

members, such as the DNA-binding domain (DBD) and the oligomerization domain (OD). Trans-activating (TA) isoforms contain an amino-terminal exon that encodes a p53-like trans-activation domain, whereas  $\Delta Np63$  isoforms lacking this domain were initially considered to be dominant-negative regulators of the TA isoforms. However, additional domains have been identified that account for transcriptional activities of the  $\Delta N$  isoforms (Dohn et al., 2001; Ghioni et al., 2002; Laurikkala et al., 2006). In addition, three alternative splicing routes at the 3' end generate TA and  $\Delta Np63$  proteins with different C-termini, denoted  $\alpha$ ,  $\beta$  and  $\gamma$ .

The p63 $\alpha$  isoforms are the most commonly expressed p63 proteins, in particular  $\Delta Np63\alpha$  is abundantly expressed in embryonic ectoderm and highly proliferative basal cells of many adult epithelial tissues including skin, breast, prostate and oral epithelium and is overexpressed in squamous cell carcinomas (Thurfjell et al., 2004).

The p53 protein is usually labile in normal cells but is dramatically stabilized upon a variety of cellular stresses. Although several ubiquitin ligases such as Pirh2, COP1 and ARF-BP1 have been reported to regulate p53 levels, MDM2 has been demonstrated to be the most important E3 ubiquitin ligase of p53 (Michael and Oren, 2002). Promoting p53 for degradation is the major mechanism by which MDM2 inhibits p53 (Itahana et al., 2007; Clegg et al., 2008).

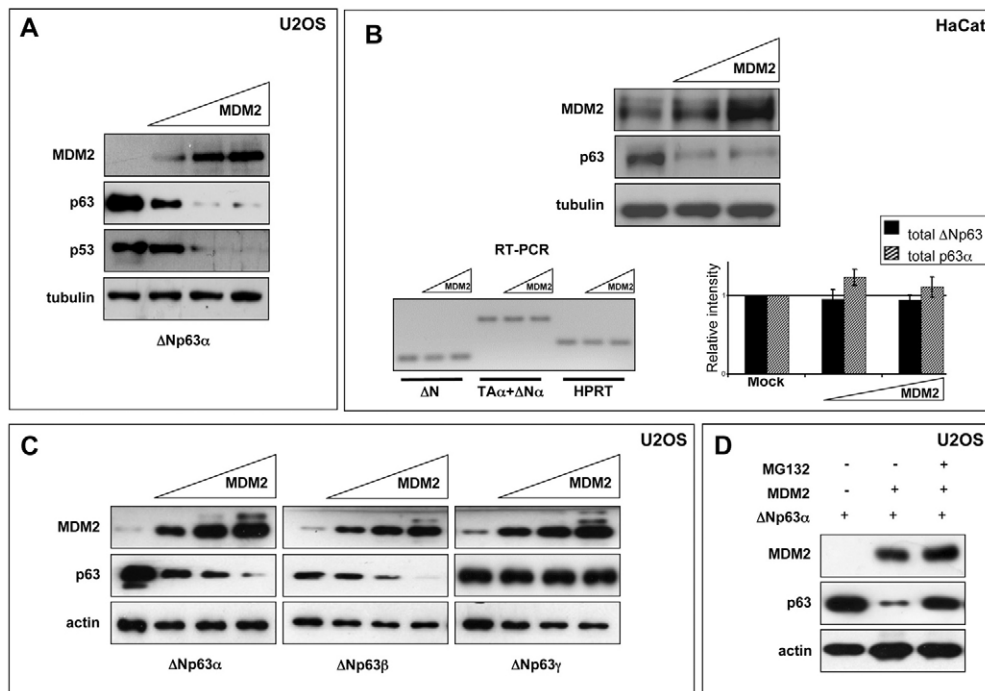
The MDM2 gene is a bona fide target downstream of the p53 gene, therefore activation of p53 upregulates its own inhibitor. MDM2 is also a p63 target but, despite the similarity between p53 and p63, the role of MDM2 in p63 protein regulation remains controversial. For instance, although we have shown that MDM2 stabilizes p63 protein and enhances its transcriptional activity in Saos2 cells (Calabrò et al., 2002), others have reported that MDM2 down-modulates p63 $\alpha$  and p63 $\gamma$  transactivation functions without affecting p63 protein levels (Kadakia et al., 2001). MDM2 was also reported to be unable to affect both p63-induced transcription and p63 protein half-life (Little and Jochemsen, 2001). All these studies, however, were performed in p53-deficient cells.

Fbw7 (also known as FBXW7) is an E3 ubiquitin ligase that interacts with the p53 pathway. It is a tumor suppressor that is mutated in various cancers (Akhoondi et al., 2007; Fuchs, 2005). Furthermore, its gene is located at chromosome 4q31.3 which is deleted in approximately 30 percent of all human cancers (Knuutila et al., 1999). It was demonstrated that p53 regulates the expression of the cytoplasmic isoform of Fbw7, Fbw7 $\beta$  (Kimura et al., 2003; Mao et al., 2004). Fbw7 might also be positioned upstream of p53 in a signaling axis that activates the tetraploidy checkpoint in response to mitotic inhibitors (Finkin et al., 2008). Moreover, Fbw7 controls the expression of central regulators of the cell cycle including cyclin E, Myc, Jun, Aurora A and the cell fate and

differentiation regulator Notch (Tan et al., 2008; Sears et al., 2000; Wei et al., 2005; Welcker and Clurman, 2008; O'Neil et al., 2007). A tight functional relationship was found between Fbw7-dependent degradation and glycogen synthase kinase 3 (GSK3) activity. Fbw7 recognition sequences, the phosphodegrons, are usually identified and bound upon phosphorylation by the GSK3 kinase (Punga et al., 2006; Welcker et al., 2003).

p63 protein stability is regulated by protein modifications such as phosphorylation, ubiquitylation and sumoylation (Ghioni et al., 2005; Rossi et al., 2006a; Rossi et al., 2006b). Proteasomes and lysosomes have both been found to be involved in p63 protein degradation (Watson and Irwin, 2006). So far, several distinct mechanisms controlling p63 protein levels have been reported. For instance, p53 was shown to be able to associate with and target  $\Delta$ Np63 into a protein degradation pathway requiring caspase 1 activity (Ratovitski et al., 2001). Recently, we have demonstrated that the homeodomain Dlx3 protein triggers proteasome-dependent  $\Delta$ Np63 $\alpha$  degradation (Di Costanzo et al., 2009) whereas  $\Delta$ Np63 $\alpha$  proteasomal degradation, in response to genotoxic stress, has been proposed to involve RACK1 (Li et al., 2009). Also, the E3 ubiquitin ligases NEDD4 and ITCH/AIP4 have both been found to be involved in the control of p63 steady state level (Rossi et al., 2006a; Rossi et al., 2006b).

In the present study, we show that MDM2 binds  $\Delta$ Np63 $\alpha$  in the nucleus promoting its translocation to the cytoplasm, where p63 is



**Fig. 1. MDM2 overexpression induces  $\Delta$ Np63 $\alpha$  and  $\Delta$ Np63 $\beta$  proteasomal mediated degradation.** (A) U2OS cells were transiently co-transfected with  $\Delta$ Np63 $\alpha$  (20 ng) and increasing amounts of MDM2 (20, 40 and 80 ng) expression vectors. 24 hours after transfection cell extracts were prepared and analyzed by western blotting (WB) with an anti-MDM2, anti-p63 or anti-p53 antibodies. Tubulin was used as a loading control. (B) Upper panel, HaCaT cells were transfected with MDM2 expression vector (50 and 100 ng). 24 hours after transfection, cell extracts were prepared and subjected to WB using anti-MDM2, anti-p63 and anti tubulin antibodies. Lower panel, plates from mock or MDM2-transfected HaCaT cells were collected for total RNA isolation. RT-PCR assays were carried out to determine the level of specific transcripts for total p63 $\alpha$  (TA $\alpha$ + $\Delta$ N $\alpha$ ) or  $\Delta$ Np63. HPRT transcript levels were determined as control. The histograms represent three independent experiments, normalized for the expression of HPRT. (C) U2OS cells were transiently co-transfected with p63 (20 ng) and MDM2 (20, 40, 80 ng) expression plasmids. 24 hours after transfection cell extracts were analyzed by WB with anti-MDM2, anti-p63 or anti-actin antibodies. (D) Extracts from U2OS cells transfected with  $\Delta$ Np63 $\alpha$  (20 ng) and MDM2 (40 ng), treated 16 hours after transfection with 10  $\mu$ M MG132 for 5 hours; p63 and MDM2 levels were evaluated by WB.

targeted for degradation by the Fbw7 E3-ubiquitin ligase. Our data suggest that, following DNA damage, MDM2 and Fbw7 can cooperate to regulate the levels of the pro-proliferative  $\Delta$ Np63 $\alpha$  protein.

## Results

### MDM2 regulates $\Delta$ Np63 $\alpha$ and $\Delta$ Np63 $\beta$ protein levels by a p53-independent mechanism

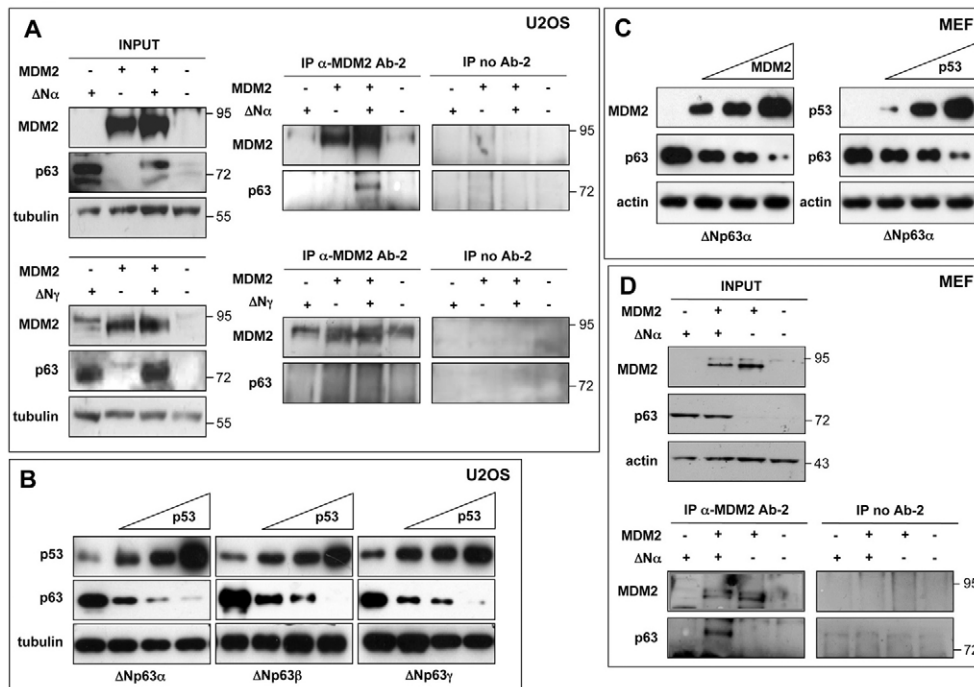
Owing to the functional interplay between p53 family members and MDM2, so far the effects of MDM2 on p63 and p73 stability have been investigated in cells lacking p53 such as the H1299 or Saos2 cells (Calabrò et al., 2002; Little and Jochemsen, 2001). We have previously shown that enforced expression of MDM2, in Saos2 cells, causes an increase of TAp63 protein levels and enhancement of its transcriptional activity (Calabrò et al., 2002). Likewise, we have recently found that all  $\Delta$ Np63 isoforms were stabilized by MDM2 overexpression in Saos2 cells (supplementary material Fig. S1). Surprisingly, we observed that co-transfection of MDM2 in U2OS cells, expressing wild-type p53, resulted in a sharp decrease of  $\Delta$ Np63 $\alpha$  protein. As expected, endogenous p53 protein was also strongly reduced by MDM2 overexpression (Fig. 1A).

To gain insight into this mechanism, we overexpressed MDM2 in HaCaT keratinocytes, a more physiological cell context, in which  $\Delta$ Np63 $\alpha$  is endogenously expressed (Di Costanzo et al.,

2009). MDM2 overexpression resulted in a dose-dependent reduction of  $\Delta$ Np63 $\alpha$  protein levels (Fig. 1B). To determine if downregulation of endogenous  $\Delta$ Np63 $\alpha$ , following MDM2 expression, was at the protein or mRNA level, we performed semi-quantitative RT-PCR analysis. As shown in Fig. 1B, MDM2-enforced expression did not result in a significant reduction of  $\Delta$ Np63-specific or p63 $\alpha$ -specific transcripts, indicating that MDM2 was acting mainly at p63 protein level.

Next, we compared the effect of MDM2 on  $\Delta$ Np63 $\alpha$ ,  $\Delta$ Np63 $\beta$  and  $\Delta$ Np63 $\gamma$  expression levels in U2OS cells. Interestingly, although the levels of  $\Delta$ Np63 $\alpha$  and  $\Delta$ Np63 $\beta$  proteins were considerably reduced,  $\Delta$ Np63 $\gamma$  was unaffected by MDM2 (Fig. 1C). Moreover, treatment of U2OS cells with the proteasome inhibitor MG132 appeared to reverse MDM2-mediated  $\Delta$ Np63 $\alpha$  degradation (Fig. 1D), while neither MDM2 nor p63 protein levels were affected by treatment with chloroquine, a lysosome-specific inhibitor (data not shown), thus suggesting that MDM2-mediated p63 degradation was proteasome-dependent.

Next, we performed co-immunoprecipitation (co-IP) experiments on U2OS cells, by transfecting  $\Delta$ Np63 $\alpha$  and  $\Delta$ Np63 $\gamma$  alone or together with MDM2. In both cases, cells were treated with MG132 in order to minimize protein degradation. Equal amount of cell extracts were immunoprecipitated with antibodies against the MDM2 protein. As shown in Fig. 2A,  $\Delta$ Np63 $\alpha$  was present in MDM2 immunocomplexes, whereas  $\Delta$ Np63 $\gamma$  was not. Remarkably,



**Fig. 2. MDM2 regulates  $\Delta$ Np63 $\alpha$  levels by a p53-independent mechanism.** (A) Extracts from U2OS cells transfected with  $\Delta$ Np63 $\alpha$  or  $\Delta$ Np63 $\gamma$  with or without MDM2 expression vector (1  $\mu$ g each), were analyzed by immunoprecipitation (IP) with an anti-MDM2 antibody followed by WB analysis with the same antibody or p63 antibody. As a control, equal aliquots of extracts were processed for IP, without addition of antibodies (IP no Ab-2 lanes). Cells were treated with 5  $\mu$ M MG132 for 5 hours in order to reduce protein degradation. The input lanes contain 5% of the material used in IP. Anti-tubulin antibodies were used as loading controls. (B) Extracts from U2OS cells co-transfected with  $\Delta$ Np63 $\alpha$ ,  $\Delta$ Np63 $\beta$  or  $\Delta$ Np63 $\gamma$  (20 ng each) and p53 (20, 40, 80 ng) were analyzed by WB using anti-p53, p63 and tubulin antibodies. (C) MEF cells (*p53*<sup>-/-</sup> and *MDM2*<sup>-/-</sup>) were co-transfected with  $\Delta$ Np63 $\alpha$  (50 ng) and MDM2 or p53 (50, 100, 150 ng in both cases). WB analysis was performed using MDM2, p53, p63 and anti-actin antibodies. (D) Extracts from MEF cells transfected with  $\Delta$ Np63 $\alpha$  (1  $\mu$ g) with or without MDM2 expression vector (1  $\mu$ g), were analyzed by immunoprecipitation (IP) with the indicated antibodies. As control, equal aliquots of extracts were processed for IP, without addition of antibodies (IP no Ab-2 lanes). The input lanes contain 5% of the material used in IP (upper panels). Cells were treated with 5  $\mu$ M MG132 for 5 hours in order to reduce protein degradation.

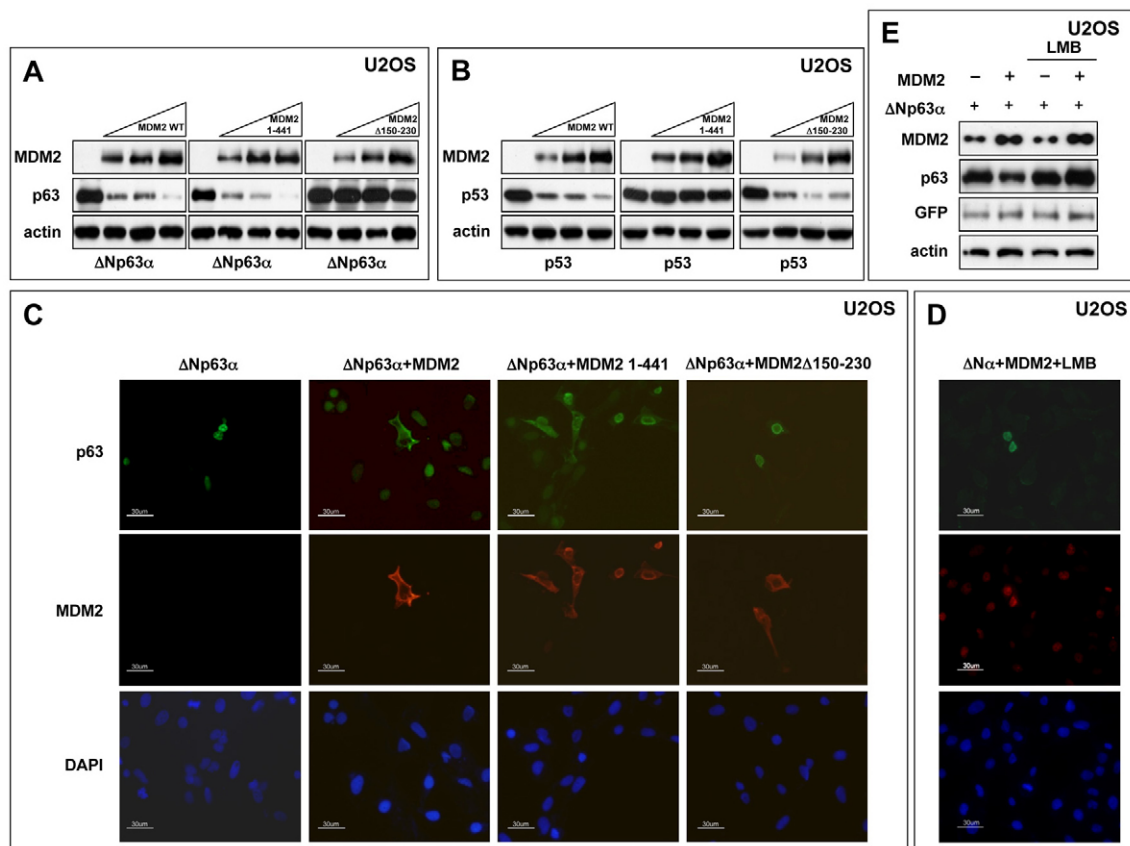
in Saos2 cells, where p63 was not regulated by MDM2, we could not detect  $\Delta$ Np63 $\alpha$ -MDM2 immunocomplexes (data not shown).

U2OS and Saos2 cells, both derived from human osteosarcomas, express similar levels of endogenous MDM2 and differ mainly in their p53 status. As p53 is a positive upstream regulator of MDM2 and was reported to induce  $\Delta$ Np63 $\alpha$  degradation (Ratovitski et al., 2001), it was of interest to evaluate the effects of p53 overexpression on  $\Delta$ Np63 isoforms in the U2OS cell line. Interestingly, expression of all  $\Delta$ Np63 proteins, including  $\Delta$ Np63 $\gamma$ , was reduced by p53 expression (Fig. 2B). Moreover, in contrast to the results obtained with MDM2, p53 overexpression resulted in degradation of all  $\Delta$ Np63 isoforms also in Saos2 cells (supplementary material Fig. S2). Furthermore, in MEF cells, derived from p53 and MDM2 double knock-out mice, overexpression of either p53 or MDM2 reduced  $\Delta$ Np63 $\alpha$  protein levels (Fig. 2C) and MDM2 was shown to interact with  $\Delta$ Np63 $\alpha$  in co-IP experiments (Fig. 2D). These results demonstrate that MDM2 and p53 can promote p63 protein degradation by independent mechanisms and that MDM2 can interact with  $\Delta$ Np63 $\alpha$  in the absence of p53.

### The RING-finger domain of MDM2 is not required to promote $\Delta$ Np63 $\alpha$ nuclear export and degradation

In order to address the mechanism through which MDM2 reduces p63 levels, we used two MDM2 mutants, the 1-441/MDM2, which lacks the RING-finger domain, necessary for protein ubiquitylation and the  $\Delta$ 150-230/MDM2 lacking the nuclear localization signal (NLS) (Jin et al., 2003; Sdek et al., 2005). As shown in Fig. 3A, the  $\Delta$ 150-230/MDM2 mutant was not efficient in promoting p63 degradation but p63 degradation triggered by 1-441/MDM2 was comparable to that obtained with wild-type MDM2. As expected, the opposite was true for the p53 protein (Fig. 3B). Similar results were obtained for p63 in p53 and MDM2 double knock-out MEF cells (data not shown).

MDM2 and p53 are nuclear proteins that shuttle constantly through the nuclear pore complex (Carter et al., 2007; O'Keefe et al., 2003) and a role for MDM2 in nuclear-cytoplasmic shuttling of p63 has been reported (Kadakia et al., 2001). Immunofluorescence analysis of U2OS cells transfected with  $\Delta$ Np63 $\alpha$  alone or with wild-type or mutant MDM2 proteins showed that the 1-441/MDM2 protein leads to  $\Delta$ Np63 $\alpha$  nuclear export and



**Fig. 3. MDM2 does not require its RING-finger domain to mediate  $\Delta$ Np63 $\alpha$  nuclear export.** (A) U2OS cells were transiently co-transfected with  $\Delta$ Np63 $\alpha$  (20 ng) or with wild-type MDM2 or MDM2 $\Delta$ 150-230 plasmids (20, 40 and 80 ng); lower amounts of MDM2-1-441 plasmid were used (10, 20 and 30 ng) in order to obtain comparable levels of the MDM2 proteins. (B) U2OS cells were transiently co-transfected with p53 expression plasmid (20 ng) and increasing amount of MDM2 expression plasmids (20, 40 and 80 ng). In both cases (A and B), WB analysis was performed using MDM2, p53 or anti-p63 antibodies. Anti-actin was used to show equal loadings. (C) Immunofluorescence staining of U2OS cells transfected with  $\Delta$ Np63 $\alpha$  alone or with wild-type MDM2, MDM2-1-441 or MDM2 $\Delta$ 150-230. Nuclei were stained with DAPI. In the absence of co-transfected MDM2, p63 localized primarily to the nucleus (100% of transfected cells) whereas co-transfection with wild-type MDM2 or MDM2 1-441 resulted in cytoplasmic p63 staining (80% and 78% of co-transfected cells, respectively). However, in cells co-transfected with MDM2 $\Delta$ 150-230, p63 remained largely nuclear (95%). (D) 24 hours post-transfection, U2OS cells transfected with  $\Delta$ Np63 $\alpha$  and MDM2 were treated with leptomycin B (LMB) at 40 ng/ml, for 5 hours. Nuclei were stained with DAPI. (E) Extracts from cells treated as in D were analyzed by WB with anti-p63 and anti-MDM2 antibodies.



this was not observed with the  $\Delta 150-230$ /MDM2 that did not enter the nucleus (Fig. 3C). Treatment with leptomycin B (LMB), a drug that blocks nuclear export, prevents p63 and MDM2 nuclear export in cells co-transfected with  $\Delta Np63\alpha$  and MDM2, thus inhibiting  $\Delta Np63\alpha$  degradation (Fig. 3D,E).

These data indicate that the MDM2 shuttling function rather than its ubiquitin ligase activity is required to mediate p63 degradation. Moreover, our data suggest that in the cytoplasm a ubiquitin ligase activity other than MDM2 is probably responsible for  $\Delta Np63\alpha$  degradation.

### S383 in $\Delta Np63$ is required for MDM2-mediated p63 protein degradation

The observation that  $\Delta Np63\gamma$  was not affected by MDM2 overexpression (Fig. 1C) and did not interact with MDM2 (Fig. 2A), suggests that the C-terminal region of  $\Delta Np63$ , absent in the  $\Delta Np63\gamma$  protein, is required for MDM2-mediated interaction. To further narrow-down the region of p63 required for MDM2 action, we employed a truncated version of  $\Delta Np63$  with a stop codon inserted at position 373 (Di Costanzo et al., 2009). As shown in Fig. 4A, the  $\Delta Np63\Delta 373$  construct was not sensitive to MDM2 thus further defining the region required for  $\Delta Np63$  degradation.

Recently, we have identified two residues, serine 383 and threonine 397 (S383 and T397), contained within the  $\alpha$  and  $\beta$  p63 isoforms, as important mediators of p63 degradation induced by Dlx3 expression (Di Costanzo et al., 2009). Interestingly, the S383A mutant was found to be resistant to MDM2-mediated degradation (Fig. 4A) whereas the T397A mutant was still sensitive to MDM2 overexpression. The opposite was true for Dlx3-mediated  $\Delta Np63$  degradation (Di Costanzo et al., 2009). These two residues are centered on two predicted phosphodegrons for the Fbw7 E3 ligase (Fig. 4B) that binds to its substrates in a phosphorylation-dependent manner. As glycogen synthase kinase 3 (GSK3) phosphorylates most of the known substrates of the Fbw7 ligase, we tested the effects of the GSK3 inhibitor SB216763 on the levels of wild-type  $\Delta Np63\alpha$ , S383A or T397A mutants. Treatment of U2OS cells with SB216763 increased the level of wild-type and T397A mutant, while the S383A protein level was unaffected by this treatment, suggesting that endogenous GSK3 was regulating  $\Delta Np63\alpha$  steady-state levels (Fig. 4B) and that Fbw7 could be

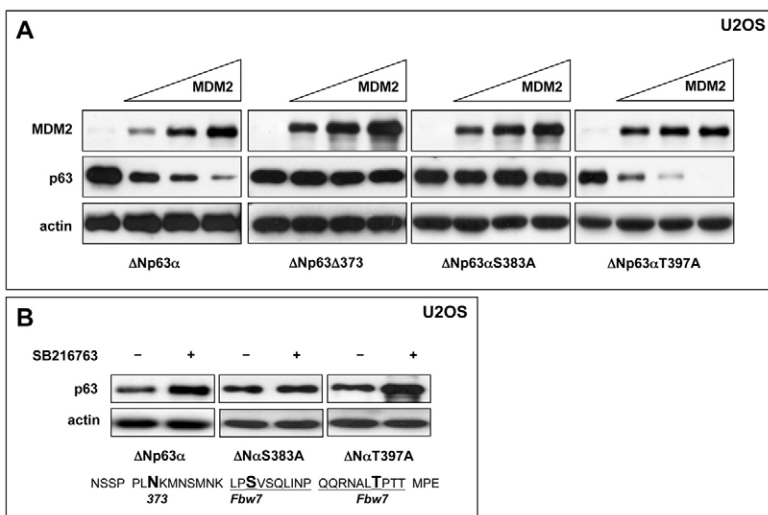
responsible for  $\Delta Np63\alpha$  degradation once exported to the cytoplasm by MDM2.

### The Fbw7 E3 ligase is responsible for MDM2-induced $\Delta Np63$ degradation

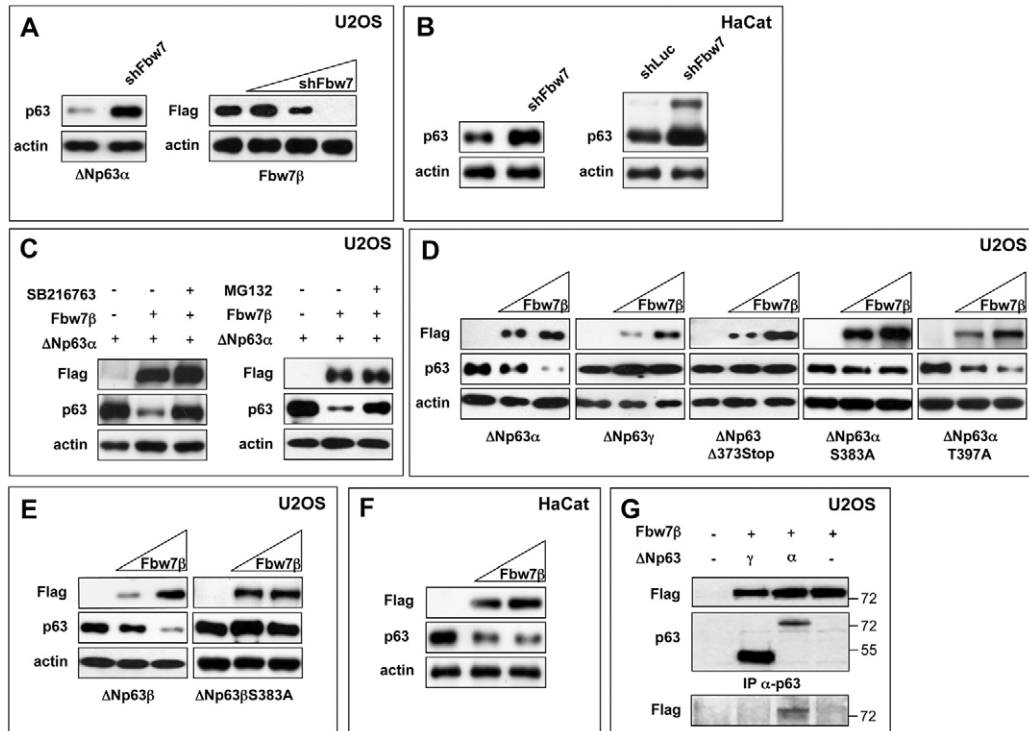
To investigate the role of Fbw7 in p63 protein turnover, we co-transfected U2OS cells with  $\Delta Np63\alpha$  and an shRNA plasmid targeting Fbw7 (Anzi et al., 2008); the levels of transfected  $\Delta Np63\alpha$  were enhanced by Fbw7 depletion (Fig. 5A). Transient or stable transfection of the shFbw7 plasmid in HaCaT cells resulted in stabilization of endogenous  $\Delta Np63\alpha$  (Fig. 5B). Conversely, co-transfection of Fbw7 with  $\Delta Np63\alpha$  resulted in the reduction of  $\Delta Np63\alpha$  levels, that was reversed upon treatment with proteasome or GSK3 inhibitors (Fig. 5C).

In perfect correlation with the ability of MDM2 to degrade wild-type and mutant p63 isoforms, Fbw7 also promoted the degradation of  $\Delta Np63\alpha$ ,  $\Delta Np63\beta$ ,  $\Delta Np63\alpha T397A$ , but not that of  $\Delta Np63\gamma$ ,  $\Delta Np63\Delta 373$ ,  $\Delta Np63\alpha S383A$  or  $\Delta Np63\beta S383A$  (Fig. 5D,E). In HaCaT cells, the endogenous levels of  $\Delta Np63\alpha$  were also modulated by Fbw7 transfection (Fig. 5F). Moreover, Fbw7 interacted with  $\Delta Np63\alpha$  but not with  $\Delta Np63\gamma$  in co-IP experiments (Fig. 5G).

The Fbw7 gene encodes three isoforms,  $\alpha$ ,  $\beta$  and  $\gamma$  that have distinct subcellular localizations, with  $\alpha$  being nuclear,  $\beta$  cytoplasmic and  $\gamma$  nucleolar. We next verified which of the Fbw7 isoforms was active on  $\Delta Np63\alpha$  by transfecting the three Fbw7 isoforms in HaCaT cells (Fig. 6A); both the Fbw7 $\alpha$  and Fbw7 $\beta$  isoforms promoted the degradation of endogenous  $\Delta Np63\alpha$ , with the Fbw7 $\beta$  isoform being more efficient in promoting  $\Delta Np63\alpha$  degradation and Fbw7 $\gamma$  having no effect on  $\Delta Np63\alpha$  protein levels. In the U2OS cell line similar results to the one obtained with overexpression of the three Fbw7 isoforms in the HaCaT cell line (Fig. 6A) were obtained; moreover, overexpression of Fbw7 did not affect MDM2 endogenous expression (Fig. 6B). Interestingly, Fbw7 $\beta$  also triggered TAp63 $\alpha$  and TAp63 $\beta$  downregulation suggesting that the same phosphodegrons are recognized by Fbw7 in the TA and  $\Delta Np63$  isoforms (supplementary material Fig. S3A). It has to be noted that our U2OS cells do not endogenously express the Fbw7 $\alpha$  isoform as revealed by RT-PCR with Fbw7-isoform-specific primers (supplementary material Fig. S3B). In accordance with the results obtained with MDM2, overexpression of the Fbw7 $\beta$



**Fig. 4. Identification of Ser383 as necessary for  $\Delta Np63$  regulation by MDM2 and Fbw7.** (A) U2OS cells were transiently co-transfected with the indicated p63 constructs (20 ng) and increasing amount of MDM2 expression plasmid (20, 40, 80 ng). WB analysis was performed on cells extracts with the indicated antibodies. (B) Bottom; schematic representation of the two Fbw7 phosphodegrons centered on S383 and T397 in  $\Delta Np63\alpha$ . Top: WB analysis of U2OS cell extracts transiently transfected with 20 ng of  $\Delta Np63\alpha$ ,  $\Delta Np63\alpha S383A$  or  $\Delta Np63\alpha T397A$ : 18 hours after transfection, cells were treated with 10  $\mu M$  SB216763 for five additional hours.



**Fig. 5. Mdm2 and Fbw7 cooperate to regulate  $\Delta$ Np63 protein levels.** (A) Left: WB analysis of U2OS cells transiently co-transfected with  $\Delta$ Np63 $\alpha$  (10 ng) and shFbw7 (20 ng) plasmids. Right, as control for the shFbw7 plasmid, U2OS cells were co-transfected with the Fbw7 expression plasmid (20 ng) and increasing amounts of shFbw7 plasmid (10, 20, 40 ng); silencing of transfected Fbw7 was visualized by anti-FLAG antibodies. (B) WB analysis of HaCaT cells transiently (left) or stably (right) transfected with the shFbw7 plasmid; endogenous  $\Delta$ Np63 $\alpha$  was stabilized upon transfection of the Fbw7 plasmid. (C) U2OS cells were transiently co-transfected with  $\Delta$ Np63 $\alpha$  (20 ng) and Fbw7 (40 ng) expression plasmids; 18 hours after transfection 10  $\mu$ M SB216763 or MG132 were added to the cultures 5 hours before harvesting. (D,E) WB analysis of U2OS cells transfected with 20 ng of the indicated  $\Delta$ Np63 $\alpha$  (D) or  $\Delta$ Np63 $\beta$  (E) constructs and increasing amounts of the Fbw7 plasmid (40 and 80 ng). (F) HaCaT cells were transfected with the Fbw7 plasmid (40 and 80 ng) and WB analysis revealed modulation of endogenous  $\Delta$ Np63 $\alpha$ . (G) Extracts from U2OS cells transfected with  $\Delta$ Np63 $\alpha$  or  $\Delta$ Np63 $\gamma$  (1  $\mu$ g) with or without Fbw7 expression vector (1  $\mu$ g), were analyzed by immunoprecipitation (IP) with an anti-p63 antibody followed by WB with the anti-FLAG antibody for Fbw7-FLAG detection (bottom panel). The input panels contain 5% of the material used in the IPs (upper panels). Cells were treated with 5  $\mu$ M MG132 for 5 hours in order to reduce protein degradation.

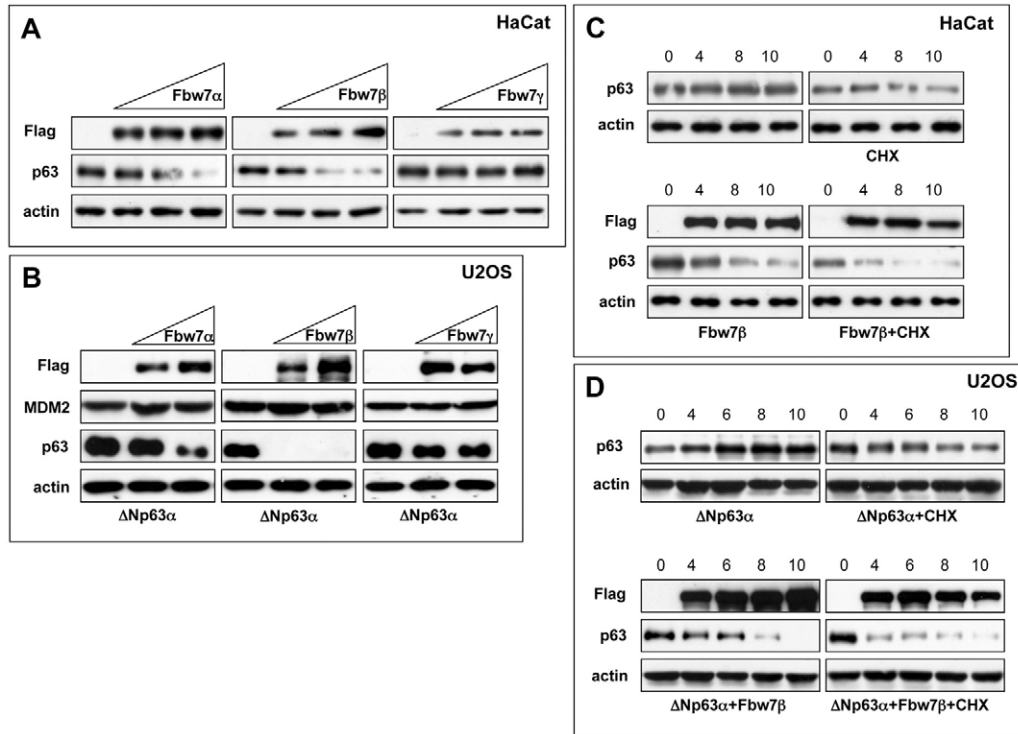
isoform in the Saos2 cell line resulted in  $\Delta$ Np63 $\alpha$  stabilization (supplementary material Fig. S4).

Finally, to prove that Fbw7 overexpression was altering  $\Delta$ Np63 $\alpha$  protein stability, we transfected Fbw7 $\beta$  into HaCaT cells and measured protein half-life by treating cells with the protein synthesis inhibitor cycloheximide. As shown in Fig. 6C, the half-life of  $\Delta$ Np63 $\alpha$  was greatly reduced by the co-transfection of Fbw7 $\beta$ , being around 6 hours in the absence of Fbw7 $\beta$  and less than 2 hours in the presence of Fbw7 $\beta$ . Very similar results were obtained in U2OS cells (Fig. 6D). Interestingly, transfection of the shFbw7 plasmid could overcome the ability of MDM2 to promote  $\Delta$ Np63 $\alpha$  degradation (Fig. 7A) and, accordingly, silencing of endogenous MDM2 by siRNA could partially overcome the action of the Fbw7 $\beta$  cytoplasmic isoform on  $\Delta$ Np63 $\alpha$  degradation (Fig. 7B). Therefore, these results demonstrate the ability of Fbw7 E3 ligase to act downstream of MDM2 in  $\Delta$ Np63 $\alpha$  degradation and that MDM2 action is required to export  $\Delta$ Np63 $\alpha$  to the cytoplasm.

Fbw7 is known to interact with its targets through the F-box domain. In order to further validate our data, we used  $\Delta$ Np63 $\alpha$  with Fbw7 $\alpha$  and Fbw7 $\beta$  expression plasmids deleted in the F-box domain in co-transfection experiments; both mutants had no effects on  $\Delta$ Np63 $\alpha$  protein levels (Fig. 7C). We next verified whether

Fbw7 overexpression could increase the levels of ubiquitylated  $\Delta$ Np63 $\alpha$  by co-transfecting U2OS cells with  $\Delta$ Np63 $\alpha$ , ubiquitin and Fbw7 $\alpha$  or Fbw7 $\beta$ . The levels of ubiquitylated  $\Delta$ Np63 $\alpha$  indeed increased in the presence of Fbw7 $\alpha$  or Fbw7 $\beta$ : as expected, in the presence of mutated Fbw7 $\alpha$  or Fbw7 $\beta$  the levels of ubiquitylated  $\Delta$ Np63 $\alpha$  did not increase (Fig. 7D).

We had previously identified lysines at position 193 and 194 in  $\Delta$ Np63 to be necessary for Itch-mediated degradation of p63 (Rossi et al., 2006b). We therefore used the double K193R/K194R mutant, in the  $\Delta$ Np63 $\alpha$  and  $\Delta$ Np63 $\beta$  isoforms, in co-transfection experiments and found that mutations of these residues in  $\Delta$ Np63 $\beta$  conferred resistance to MDM2 as well as to Fbw7 $\beta$  overexpression (Fig. 8) whereas the  $\Delta$ Np63 $\alpha$  isoform was only partially protected. Lysines 494 and 505 had been previously identified as necessary for ubiquitin-mediated degradation of  $\Delta$ Np63 $\alpha$  (F.G. and L.G., unpublished results). These two lysines are not present in the  $\Delta$ Np63 $\beta$  isoform and therefore cannot account for  $\Delta$ Np63 $\beta$  degradation. To further elucidate this point, we examined whether lysines 494 and 505 were involved in MDM2 and Fbw7 $\beta$  degradation of  $\Delta$ Np63 $\alpha$ . Remarkably, the  $\Delta$ Np63 $\alpha$ K494R/K505R mutations conferred complete resistance to MDM2- and Fbw7-mediated degradation (Fig. 8). These results suggest that K494 and K505 are involved in  $\Delta$ Np63 $\alpha$  degradation.



**Fig. 6. MDM2 and Fbw7 act in concert to regulate  $\Delta$ Np63 $\alpha$  protein levels.** (A) HaCaT cells were transiently transfected with the three Fbw7 isoforms (20, 40 and 80 ng). WB analysis revealed modulation of endogenous  $\Delta$ Np63 $\alpha$  in the presence of Fbw7 $\alpha$  and Fbw7 $\beta$ . Fbw7 $\gamma$  had no effect on  $\Delta$ Np63 $\alpha$  protein. (B) U2OS cells were transiently co-transfected with  $\Delta$ Np63 $\alpha$  and the Fbw7 isoforms. (C) HaCaT cells were transiently transfected with Fbw7 $\beta$  and protein half-life was measured upon cycloheximide (CHX) treatment either in the absence or in the presence of Fbw7, for the indicated times (numbers indicate hours after CHX addition). (D) U2OS cells were transiently transfected with  $\Delta$ Np63 $\alpha$  and protein half-life was measured upon cycloheximide (CHX) treatment either in the absence or in the presence of Fbw7 $\beta$ , for the indicated times (numbers indicate hours after CHX addition). WB analysis revealed that Fbw7 $\beta$  greatly reduced  $\Delta$ Np63 $\alpha$  protein half-life.

### MDM2 and Fbw7 cooperate to regulate $\Delta$ Np63 $\alpha$ degradation upon DNA damage and keratinocyte differentiation

$\Delta$ Np63 $\alpha$  has been shown to be degraded upon exposure of cells to UV and adriamycin (Papoutsaki et al., 2005). In order to gain insight into the physiological mechanisms involved in the regulation of  $\Delta$ Np63 $\alpha$  protein levels by the MDM2-Fbw7 pathway, we examined whether knockdown of Fbw7, by shRNA, or MDM2, by siRNA oligonucleotides, could inhibit degradation of  $\Delta$ Np63 $\alpha$  in HaCaT cells treated with UV. As shown in Fig. 9A,B, UV exposure caused downregulation of endogenous  $\Delta$ Np63 $\alpha$  protein levels, and silencing of endogenous MDM2 or Fbw7 blocked the degradative process. Similar results were obtained in U2OS cells co-transfected with  $\Delta$ Np63 $\alpha$  and the shFbw7 plasmid upon UV exposure (Fig. 9C) or adriamycin treatment (supplementary material Fig. S5). The levels of the endogenous MDM2 protein were not altered by Fbw7 silencing in both cell lines (Fig. 9C and not shown). As controls, siLuc or shLuc were used.

$\Delta$ Np63 $\alpha$  has also been shown to be degraded upon keratinocyte differentiation (Di Costanzo et al., 2009). In order to verify whether MDM2 and Fbw7 could regulate  $\Delta$ Np63 $\alpha$  protein levels during this process as well, pools of clones of HaCaT cells stably silenced for Fbw7 expression were induced to differentiate by the addition of 2 mM  $\text{Ca}^{2+}$  to the culture medium. In the control clones, stably transfected with the shLuc plasmid,  $\Delta$ Np63 $\alpha$  was downregulated after 24 hours and upregulation of keratin-1, a differentiation

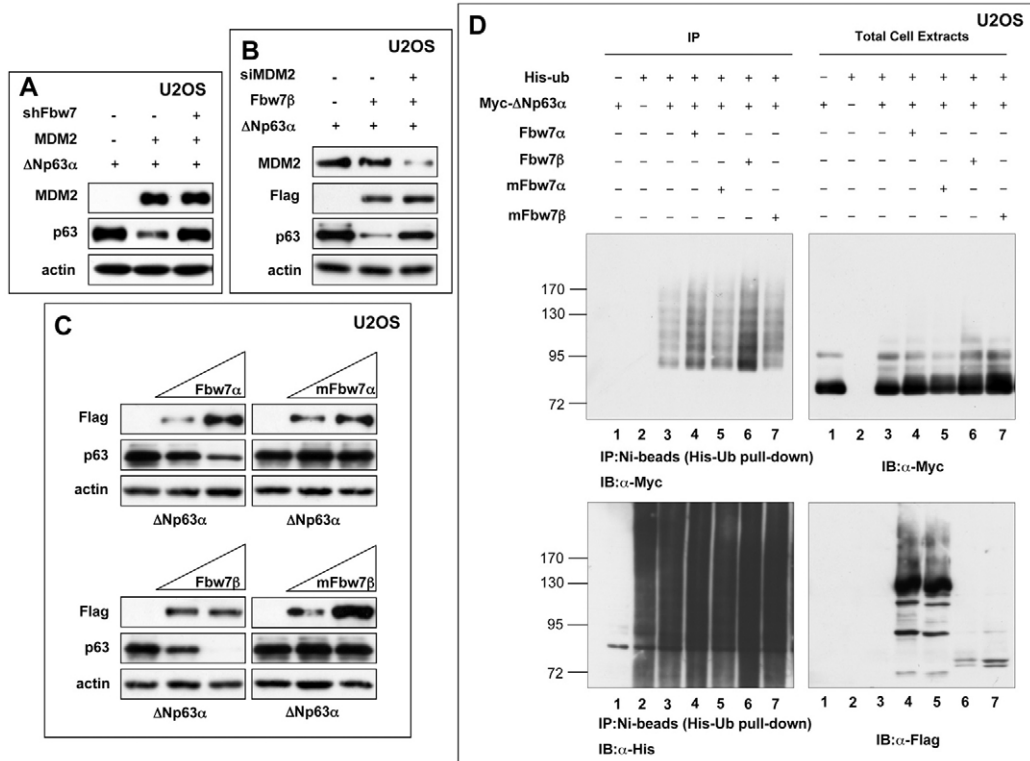
marker, was also evident. In the Fbw7-silenced clones, both  $\Delta$ Np63 $\alpha$  downregulation and keratin-1 upregulation were impaired (Fig. 9D).

These data suggest that, under stress conditions that might lead to cell cycle arrest or apoptosis and during cell differentiation, MDM2 and Fbw7 act in concert to trigger  $\Delta$ Np63 $\alpha$  degradation.

### Discussion

Protein degradation is a major regulatory mechanism of all cellular functions. This process is associated in most cases with labeling of the target protein with a low molecular mass protein, ubiquitin. The Fbw7 protein is an F-box factor that determines which proteins will be targeted for degradation by ubiquitin ligation. Fbw7 targets for degradation a long list of proteins with central regulatory roles in cell division, cell growth and differentiation, including Myc, Jun and Notch. Its activity is essential for preservation of genomic stability and for prevention of tumor formation. In fact, the Fbw7 gene is mutated in a wide spectrum of human cancers, thus suggesting that it is a bona fide tumor suppressor. Numerous cancer-associated mutations in Fbw7 and its substrates have been identified, and loss of Fbw7 function causes chromosomal instability and tumorigenesis.

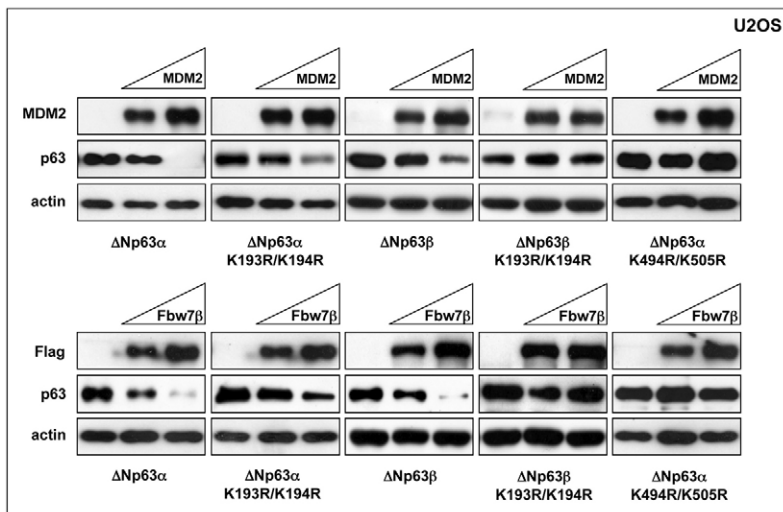
Because there are three Fbw7 isoforms that reside in different subcellular compartments, as well as multiple Fbw7 substrates that are the products of proto-oncogenes, the mechanisms of tumor suppression by Fbw7 are complex and not completely understood.



**Fig. 7. Ubiquitylation of  $\Delta$ Np63 $\alpha$  is enhanced by Fbw7 $\alpha$  and Fbw7 $\beta$  in vivo.** (A) WB analysis of U2OS cells transiently transfected with  $\Delta$ Np63 $\alpha$  (20 ng) and MDM2 (40 ng) expression plasmids with or without the shFbw7 plasmid (40 ng). (B) WB analysis of U2OS cells transfected with  $\Delta$ Np63 $\alpha$  (20 ng) and Fbw7 $\beta$  (40  $\mu$ M) with or without siRNA oligonucleotides targeting MDM2 (5 nM). (C) WB analysis of U2OS cells transiently co-transfected with  $\Delta$ Np63 $\alpha$  (20 ng) and wild-type or mutated Fbw7 $\alpha$  and Fbw7 $\beta$  (20 and 40 ng) expression plasmids. (D) U2OS cells were transiently transfected with the indicated expression plasmids: His-ubiquitin (1  $\mu$ g), Myc- $\Delta$ Np63 (2  $\mu$ g), FLAG-Fbw7 $\alpha$  and FLAG-Fbw7 $\beta$  (4  $\mu$ g), mutant Fbw7 $\alpha$  and Fbw7 $\beta$  (4  $\mu$ g). 24 hours later, cells were treated with MG132 (20  $\mu$ M, 4 hours) and lysed under denaturing conditions. Proteins linked to His-ubiquitin were purified using Ni<sup>2+</sup>-resin beads, washed and eluted with 200 mM imidazole. His-ubiquitin-p63 conjugates were detected by western blotting using anti-Myc antibody (upper left panel). As control, total His-ubiquitin conjugates were monitored by anti-His antibody (Sigma, lower left panel). The amount of Myc-p63 and the FLAG-tagged Fbw7 constructs in the total cell extract were determined by blotting the membrane with anti-Myc and anti-FLAG antibodies, respectively (right panels).

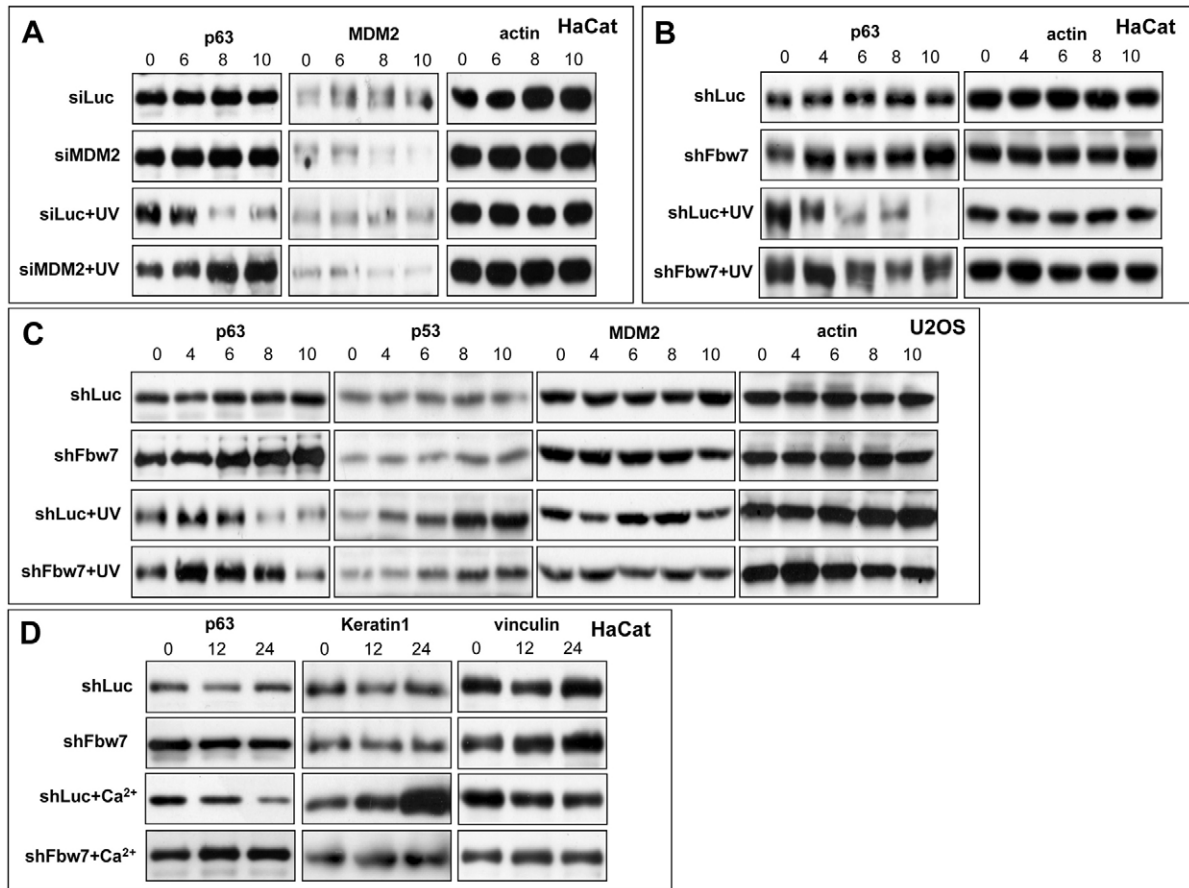
Here, we show that MDM2 and Fbw7 cooperate in the regulation of  $\Delta$ Np63 protein stability in human tumor cell lines as well as in HaCaT keratinocytes, a more physiological cell context in which  $\Delta$ Np63 $\alpha$  is endogenously expressed. Our immunofluorescence and western blot data with wild-type and mutant MDM2 proteins

indicate that MDM2 associates with and shuttles  $\Delta$ Np63 $\alpha$  from the nucleus to the cytoplasm. Moreover, our ubiquitylation assays in U2OS cells, show that Fbw7 coexpression increases the levels of ubiquitylated  $\Delta$ Np63. By overexpression and silencing approaches, we demonstrate that Fbw7 $\beta$  is acting downstream of MDM2 in



**Fig. 8. Efficient regulation by MDM2 and Fbw7 requires different lysines in  $\Delta$ Np63 $\alpha$  and  $\Delta$ Np63 $\beta$ .** WB analysis of U2OS cells transiently co-transfected with the indicated p63 (20 ng), MDM2 or Fbw7 (40 and 80 ng) expression plasmids.





**Fig. 9. Fbw7 induces  $\Delta$ Np63 $\alpha$  degradation upon DNA damage.** (A,B) HaCaT cells were transiently transfected with siRNA-MDM2 oligonucleotides (5 nM) or with shFbw7 plasmid (20 ng) or shLuc (20 ng). After 12 hours, cells were exposed to 100  $\mu$ J UV. Cells were collected at the indicated time points (hours) and cell extracts were analyzed by western blotting. (C) U2OS cells were transiently transfected with  $\Delta$ Np63 $\alpha$  (20 ng) with or without the shFbw7 plasmid (40 ng): 12 hours after transfection, cells were exposed to 100  $\mu$ J UV. Cells were collected at the indicated time points (hours) and extracts were analyzed by WB analysis. The levels of endogenous p53 activation were evaluated as controls for the treatments. (D) Stable pools of HaCaT cells were induced to differentiate by addition of 2 mM Ca<sup>2+</sup> to the culture medium. 12 and 24 hours after Ca<sup>2+</sup> addition, cell extracts were collected and analyzed by western blotting.

this mechanism. Interestingly, the activity of GSK3, the kinase known to phosphorylate Fbw7 recognition sites, is required for this mechanism.

DNA-damaging agents exert opposite effect on p53 and  $\Delta$ Np63 $\alpha$ ; downregulation of  $\Delta$ Np63 $\alpha$  while activating p53 (Petitjean et al., 2006). This is not surprising since it has been proposed that the  $\Delta$ Np63 $\alpha$  oncogenic role is based exactly on its ability to counteract the p53 transcriptional response to DNA damage, by competing for DNA binding to common target promoters (Crook et al., 2000; Murray-Zmijewski et al., 2006). The pro-proliferative  $\Delta$ Np63 $\alpha$  protein has also been shown to be downregulated upon keratinocytes differentiation (Di Costanzo et al., 2009).

Remarkably, by silencing experiments, we have demonstrated that both MDM2 and Fbw7 contribute to reduce the endogenous or transfected  $\Delta$ Np63 $\alpha$  protein levels when cells are treated with DNA-damaging agents or upon cellular differentiation. Interestingly, it has been reported that exposure to UV induces the expression of the cytoplasmic Fbw7 $\beta$  isoform (Anzi et al., 2008). Among the three Fbw7 isoforms, we found that the cytoplasmic Fbw7 $\beta$  and nuclear Fbw7 $\alpha$  isoforms are active on  $\Delta$ Np63 $\alpha$ , although Fbw7 $\beta$  was more efficient. Interestingly, the  $\alpha$ -isoform is

not expressed in U2OS cells. Further experiments will be necessary to determine the physiological contexts in which the activity of one or the other isoform becomes relevant.

p53 and MDM2 are both able to induce  $\Delta$ Np63 $\alpha$  degradation (Ratovitski et al., 2001) (this work), although by different and independent mechanisms. These observations seem to be contradictory as it is known that p53 and MDM2 exert antagonistic effects on cell proliferation and transformation. Actually, we and others (Kadakia et al., 2001) have found that MDM2 is able to mediate  $\Delta$ Np63 $\alpha$  export to the cytoplasm. Accordingly, we propose that, under appropriate stimuli, such as DNA damage that lead to cell cycle arrest or apoptosis or cellular differentiation, MDM2 can shuttle  $\Delta$ Np63 $\alpha$  to the cytoplasm where it can encounter the proteins that are specifically involved in its turnover, such as Fbw7 (this work) and Itch (Rossi et al., 2006a; Rossi et al., 2006b). At the same time, removal of  $\Delta$ Np63 $\alpha$  from the nuclear compartment, allows p53 to exert its transcriptional activities. As reported, p53 might finely regulate Fbw7 $\beta$  and MDM2 expression that thereafter cooperate in regulating the  $\Delta$ Np63 $\alpha$  protein level. However, it is conceivable that, under apoptotic levels of DNA damage, p53 could directly regulate  $\Delta$ Np63 $\alpha$  levels through caspase-1 activity (Ratovitski et al., 2001). According to this model, our preliminary

experiments indicate that the caspase-1-resistant  $\Delta$ Np63 $\alpha$  mutant (YVED to YVEA) is still responsive to the cooperative MDM2 and Fbw7 degradation mechanism (F.G. and L.G., unpublished data).

In epithelia,  $\Delta$ Np63 $\alpha$  supports the proliferative potential of basal cells and its downregulation is required for keratinocytes differentiation (Koster et al., 2002; Koster et al., 2004). Several mechanisms have been reported to govern  $\Delta$ Np63 $\alpha$  levels, none of them involving MDM2. Interestingly, a striking and transient induction of MDM2 has been reported in the suprabasal layers of stratified epithelia, whereas overexpression of MDM2 in the basal layers has been reported to cause skin hyperplasia (Ganguli et al., 2000). Altogether these observations and our data suggest that both MDM2 and Fbw7 cooperate in order to finely tune  $\Delta$ Np63 $\alpha$  levels during epithelia differentiation.

An outstanding question in the field of p63 epithelial biology is whether, and to what extent,  $\Delta$ Np63 $\alpha$  can contribute to tumor growth and response to anticancer agents. The hypothesis that  $\Delta$ Np63 $\alpha$  plays a role in the genesis and progression of tumors is supported by the findings that  $\Delta$ Np63 $\alpha$  is upregulated in human ovarian cancer and squamous cell carcinomas (SCC) (Thurfjell et al., 2004; Deyoung and Ellisen, 2007). Since the molecular mechanisms leading to  $\Delta$ Np63 overexpression are still unknown, our data open a new way to future studies to correlate Fbw7 mutations with  $\Delta$ Np63 $\alpha$  overexpression. Most of the natural Fbw7 mutations falls within the F-box domain, the integrity of which we have now shown to be necessary to promote  $\Delta$ Np63 $\alpha$  degradation and ubiquitylation.

The knowledge of the role of p63, MDM2 and Fbw7 in the molecular mechanisms governing the response of cancer cells to DNA damage is crucial for improving current anti-cancer therapies (Koster et al., 2004; Ganguli et al., 2000).

## Materials and Methods

### Plasmids

All expression vectors encoding p63 cDNAs have been previously described (Ghioni et al., 2002). The MDM2, Fbw7, shFbw7,  $\Delta$ Np63 $\alpha$ S383A and  $\Delta$ Np63 $\alpha$ T397A constructs also have been previously described (Jin et al., 2003; Anzi et al., 2008; Di Costanzo et al., 2009). The Fbw7 mutant plasmids having a deletion of the conserved five amino acids right at the beginning of the F-box domain (LPKEL) were a kind gift from B. E. Clurman and M. Welcker (Fred Hutchinson Cancer Research Center, Seattle, WA).

The  $\Delta$ Np63 $\alpha$ K193R/K194R,  $\Delta$ Np63 $\alpha$ K494R/K505R and  $\Delta$ Np63 $\beta$ K193R/K194R mutants were generated by site-directed mutagenesis with the following mutagenic oligonucleotides. K193R-K194R: forward 5'-GCCATGCCTGTCTACAGGAGAGCCTCAGCATGTCACC-3', reverse 5'-GGTGACATGCTCAGCTCCTGTAGACAGGCATGGC-3'; K494R: forward 5'-GGCAAGTCTGAGGATCCCTGAA-CAGTTCGACATGCCATCTGG-3', reverse 5'-CCAGATGGCATGTCGGAACCTGTCAGGCATCCTCAGACTTGGC-3'; K505R: forward 5'-CCGACATGCC-ATCTGGAGGGGCATCCTGGACCACAGG-3', reverse 5'-CCTGTGGTCCAG-GATGCCCTCCAGATGGCATGTCGG-3'.

### Cell culture and transfection

Saos-2, U2OS, MEF and HaCaT cells were maintained in DMEM supplemented with 10% FBS (Gibco) at 37°C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub> in air. For transient transfection, 50,000 cells were seeded into 24-well multiplates and on the next day transfected with Lipofectamine 2000 (Invitrogen) or Lipofectamine LTX (Invitrogen) for HaCaT cells, under the conditions suggested by the manufacturer. Transfection efficiency was always monitored in separate wells by transfection of a  $\beta$ -gal expression vector. The total amount of transfected DNA (1  $\mu$ g) was kept constant using empty vector when necessary.

For stable transfection, 300,000 HaCaT cells were seeded into six-well multiplates in DMEM supplemented with 10% FBS (Gibco). On the next day, cells were co-transfected with 3  $\mu$ g of shFbw7 and 3  $\mu$ g pSuperRetro or 3  $\mu$ g of shLuc and 3  $\mu$ g pSuperRetro using Lipofectamine LTX (Invitrogen). After 24 hours, cells were trypsinized and plated in medium containing puromycin (0.8  $\mu$ g/ml; Sigma). After 3 weeks of selection, clones were pooled and kept in puromycin (0.4  $\mu$ g/ml). For the differentiation assay, 50,000 cells were plated in 24-well plates and, on the following day, induced to differentiate by the addition of Ca<sup>2+</sup> (2 mM) to the culture medium. U2OS and HaCaT cells were treated with 5 or 10  $\mu$ M MG132, 100  $\mu$ M chloroquine,

40 ng/ml leptomycin B, 10  $\mu$ M cycloheximide, 2  $\mu$ M adriamycin, 10  $\mu$ M SB216763 or irradiated with 100  $\mu$ J. Stable pools of HaCaT clones were induced to differentiate by addition of 2 mM Ca<sup>2+</sup> to the culture medium.

The sequences of the siRNA were as follows: siMDM2, 5'-AAG CCA UUG CUU UUG AAG UUA TT-3'; siLuc, 5'-CGU ACG CGG AAU ACU UCG ATT-3'.

### Western blot analysis and antibodies

24 hours after transfection, cells were lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 5 mM EDTA, 0.5% sodium deoxycholate, 1  $\mu$ M phenylmethylsulfonyl fluoride and protease inhibitors. Cell lysates were incubated on ice for 30 minutes and the extracts were centrifuged at 6600 g for 10 minutes to remove cell debris. Protein concentration was determined with the Bio-Rad protein assay. Samples were resuspended in 2 $\times$  loading buffer (Sigma), incubated at 95°C for 10 minutes and resolved by SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore). The blots were incubated with the following antibodies: p63 (4A4 and H-129, Santa Cruz Biotechnology), p53 (p53 DO-1, Genespin, Italy), MDM2 (SMP14, Santa Cruz Biotechnology; Ab-2, Calbiochem),  $\beta$ -tubulin (H-235, Santa Cruz Biotechnology), actin (A-2066, Sigma-Aldrich), vinculin (V 9131, Sigma-Aldrich), FLAG (M2, Sigma). Proteins were visualized by an enhanced chemiluminescence method (Genespin) according to the manufacturer's instructions.

### Co-immunoprecipitation

U2OS, MEF-p53-MDM2 double knockout and Saos2 cells (1.25 $\times$ 10<sup>6</sup>/100 mm plate) were transfected with the indicated vectors. 24 hours after transfection cells were harvested for preparation of whole-cell lysates as described above. 1 mg/ml of cell lysate was incubated overnight at 4°C with 3  $\mu$ g of anti-p63 (4A4, H137 or H-129; Santa Cruz, Biotechnology) or anti-MDM2 (Ab-2, Calbiochem). The immunocomplexes were collected by incubating with protein-A-agarose (Roche) at 4°C for 4 hours. The beads were washed vigorously three times with coimmunoprecipitation buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.5% NP40; 10% glycerol). The beads were then resuspended in 2 $\times$  loading buffer, loaded directly onto an 8% SDS polyacrylamide gel and subjected to western blot with the indicated primary antibodies.

### Immunofluorescence

For immunofluorescence staining, U2OS cells were plated onto glass cover slips before transfection. 24 hours after transfection, the cells were fixed with 3.7% paraformaldehyde in PBS for 15 minutes at room temperature, and permeabilized by dipping coverslips in 0.5% Triton X-100 in PBS for 15 minutes. Coverslips were washed three times with PBS and then incubated for 1 hour at room temperature with primary antibodies. p63 staining was carried out using the anti-p63 H-137 antibody (Santa Cruz, Biotechnology), and MDM2 staining was carried out using the anti-MDM2 monoclonal antibody Ab-2 (Calbiochem). After three washes in PBS, cells were incubated for 1 hour with secondary antibody [Cy3-labeled goat anti-mouse (Jackson Lab) or fluorescein-labeled anti-rabbit (Roche)]. Nuclei were stained with DAPI (Sigma).

### RNA extraction and RT-PCR

24 hours post-transfection, HaCaT cells were collected and total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For RT-PCR analysis, 600 ng of total RNA were reverse-transcribed using SuperscriptIII cDNA Preparation Kit (Life Technologies) with random hexamer primers and the cDNAs were normalized to HPRT levels.

For PCR reaction the sequence of the primers pairs were as follows.  $\Delta$ Np63 $\alpha$ ,  $\Delta$ Np63 $\beta$  and  $\Delta$ Np63 $\gamma$ : forward 5'-GAAGAAAGGACAGCAGCATTGAT-3', reverse 5'-GGGACTGGTGGACGAGGAG-3';  $\Delta$ Np63 $\alpha$  (TA and  $\Delta$ Np63): forward 5'-GGGAGCCAACATTCCCATGAT-3', reverse 5'-ACTTGCCAGATCATCCA-TGG-3'; HPRT: forward 5'-AAGCCAGACTTTGTTGGATTT-3'; reverse 5'-TTTACTGGCGATGTCAATAGGA-3'; Fbw7 $\alpha$ : forward 5'-GACCTCAGA-ACCATTGGTCCAACCT-3', reverse 5'-AGTAGTATGTGGACCTGGCCCGTT-3'; Fbw7 $\beta$ : forward 5'-GACCTCAGAACCATTGGTCCAACCT-3', reverse 5'-TATTGTGACAGACTGCCAAGCAGC-3'; Fbw7 $\gamma$ : forward 5'-GCCTTGGG-CAATGATGCTAATGCT-3', reverse 5'-CCATGGCTTGGTTCCTGTTGATC-3'.

PCR products were analyzed on 2% agarose gel followed by ethidium bromide staining.

### In vivo ubiquitylation assay

The in vivo ubiquitylation assay was been done as previously described (Louria-Hayon et al., 2009) with the only exception, that the lysis was performed with 10 mM imidazole (not 20 mM).

We thank A. Costanzo for critical reading of the manuscript. We also thank Bruce E. Clurman and Markus Welcker (Fred Hutchinson Cancer Research Center) for the generous gift of the Fbw7 mutant plasmids. L.G. was supported by Telethon (GGP05056) and PRIN. G.L.M. was funded by AIRC and PRIN. M.R. was supported by an AIRC fellowship. E.S. was supported by grant number 492/05 from

the Israel Academy of Science. Y.H. was supported by the NHMRC project grants (grant no. 50916, 509197) and by the VESKI award.

Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/14/2423/DC1>

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