Skp2B attenuates p53 function by inhibiting prohibitin

Harish Chander¹, Max Halpern¹, Lois Resnick-Silverman², James J. Manfredi² & Doris Germain^{1*}

¹Division of Hematology and Medical Oncology, Department of Medicine, and ²Department of Oncological Sciences, Mount Sinai School of Medicine, New York, USA

The F-box protein Skp2 and its isoform Skp2B are both overexpressed in breast cancers. Skp2 alters the activity of p53 by inhibiting its interaction with p300 and by promoting p300 degradation. Here, we report that Skp2B also attenuates the activity of p53; however, this effect is independent of p300, suggesting that another mechanism might be involved. Prohibitin, a protein reported to activate p53, was isolated in a two-hybrid screen with the carboxy-terminal domain unique to Skp2B. We observed that prohibitin is a new substrate of Skp2B and that the degradation of prohibitin is responsible for the attenuated activity of p53 in cells overexpressing Skp2B. Furthermore, we show that the activity of p53 is reduced in the mammary glands of Skp2B transgenic mice. This study indicates that both Skp2 and Skp2B attenuate p53 activity through different pathways, suggesting that amplification of the Skp2 locus represents a powerful mechanism to attenuate p53 function in cancer.

Keywords: p53; prohibitin; ubiquitin pathway

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INTRODUCTION

F-box proteins function as the substrate recognition subunits of specific ubiquitin ligase complexes. Skp2 is one of the best-characterized F-box proteins and has been implicated in the degradation of the cyclin-dependent kinase inhibitor p27. Skp2 was recently shown to attenuate p53 function by affecting the activity and level of the acetyltransferase enzyme p300 (Kitagawa *et al*, 2008). Skp2B is an isoform of Skp2 that differs at the carboxy-terminal domain and is important for substrate recognition. As a result, Skp2B does not show a significant effect on Skp2 substrates (Radke *et al*, 2005). Both Skp2 and Skp2B are overexpressed in breast cancer.

To determine whether Skp2B does have a role in breast cancer, we established transgenic mice overexpressing Skp2B in the mammary gland under the control of the mouse mammary tumour virus (MMTV) promoter (Umanskaya *et al*, 2007). We found that MMTV-Skp2B mice develop several phenotypes including

mammary tumours (Umanskaya *et al*, 2007), but the substrate of Skp2B involved in its oncogenic effect remained unidentified. Here, we report that prohibitin, a protein reported to activate p53, is targeted for degradation upon Skp2B overexpression and that as a result, the activity of p53 is attenuated both *in vitro* and *in vivo*.

RESULTS

Skp2B affects prohibitin levels

Both Skp2 and Skp2B are overexpressed in breast cancer (Radke *et al*, 2005). Skp2 attenuates p53 function (Kitagawa *et al*, 2008) but whether Skp2B can also attenuate p53 has not yet been determined. We found that while MCF-7 cells (low Skp2 and Skp2B) and HBL-100 cells (high Skp2 and Skp2B; Fig 1A) are both wild-type for p53, its activity is significantly lower in HBL-100 cells (Fig 1A). To determine whether Skp2B contributes to this effect, a p53-luciferase reporter was transfected in MCF-7 cells and cells overexpressing Skp2B, which were then treated with 10 μ M of camptothecin. We observed that p53 activity was reduced markedly in cells overexpressing Skp2B (Fig 1B). We next tested whether Skp2B affects p300 by using MCF-7 cells overexpressing Skp2 as a positive control. As reported previously, p300 levels were reduced in cells overexpressing Skp2 but remained unchanged in Skp2B cells (supplementary Fig S1A online), confirming previous observations that Skp2B does not affect the substrates of Skp2. However, this result indicates that the attenuation of p53 activity in Skp2B cells is due to other substrates. We previously performed a two-hybrid screen to identify potential substrates of Skp2B by using the carboxy-terminal domain, which is unique to Skp2B, as the bait. The screen identified prohibitin but the interaction was not confirmed. However, as prohibitin has been reported to have a role in the activation of p53 (Fusaro *et al*, 2003), it is a potential candidate for the effect of Skp2B on p53 activity; therefore, we first validated the interaction between endogenous Skp2B and endogenous prohibitin by using immunoprecipitation. An interaction between endogenous Skp2B and prohibitin was detected under conditions in which Skp2B is stabilized by using the proteasome inhibitor LLnL in MCF-7 cells or in HBL-100 cells that express elevated levels of Skp2B (Fig 1C). However, as the antibody used for western blotting recognized both Skp2 and Skp2B, we then aimed at distinguishing between Skp2 and Skp2B by using cell lines that express Skp2-Flag or Skp2B-Flag. We observed that prohibitin immunoprecipitates with Skp2B but not with Skp2, even if Skp2 is more abundant than Skp2B (Fig 1D). To determine the effect of Skp2B on prohibitin,

¹Division of Hematology and Medical Oncology, Department of Medicine, and

²Department of Oncological Sciences, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, Box 1079, New York 10029, USA

*Corresponding author. Tel: +1 212 241 9541; Fax: +212 876 7299;

E-mail: doris.germain@mssm.edu

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Fig 1 | Skp2B affects the level of prohibitin. (A) MCF-7 and HBL-100 breast cancer cells were transfected with 1 μ g of *p53* reporter and after 24 h the *p53* activity was determined. (B) MCF-7 and MCF-7-Skp2B cells were transfected with *p53* reporter and after 24 h camptothecin (10 μ M) was added. The activity of *p53* was measured after 24 h. (C) MCF-7 cells treated with 50 μ M of LLnL and untreated HBL-100 cells were used for immunoprecipitation with either a control IgG or prohibitin antibody, followed by western blot analysis with a Skp2/B antibody. Inputs of Skp2/B levels in crude lysates are shown below. (D) Immunoprecipitation of endogenous prohibitin in MCF-7 cells expressing either Skp2-Flag or Skp2B-Flag at low levels. (E) Western blot analysis of prohibitin and Skp2B in MCF-7 cells, MCF-7-Skp2B cells and MCF-7 cells transfected with 10 nM of siRNA against Skp2B. Tubulin was used as a loading control. The numbers below each blot represent the Skp2B/tubulin and prohibitin/tubulin ratio, respectively. IB, immunoblotting; IgG, immunoglobulin; IP, immunoprecipitation; LLnL, leuciny-leuciny-norleuciny; mRNA, messenger RNA; siRNA, small interfering RNA.

MCF-7-Skp2B and MCF-7 cells, transfected with small interfering RNA (siRNA) against Skp2B (Fig 1E), were used to measure the levels of prohibitin by western blot analysis. We observed that the level of prohibitin decreased 10-fold in cells overexpressing Skp2B, whereas it was elevated 1.7-fold on inhibition of endogenous Skp2B by siRNA (Fig 1E). The same result was obtained with a second siRNA against Skp2B (supplementary Fig S1B online). This indicates that although endogenous Skp2B has a mild effect on prohibitin levels, Skp2B overexpression markedly reduces prohibitin. In addition, we found that overexpression of a mutant of Skp2B, lacking the C-terminus (Skp2BAC), had no effect on the level of prohibitin (supplementary Fig S1C online). To determine further the specificity of Skp2B on prohibitin, we analysed its level in a stable clone overexpressing Skp2. The overexpression of Skp2 had no effect (supplementary Fig S1D online). These results indicate that levels of prohibitin are affected specifically by Skp2B and that its C-terminal domain is required for the interaction.

Skp2B promotes the degradation of prohibitin

As Skp2B acts as a component of the Skp1-cullin-F-box ubiquitin ligase complex (Radke *et al*, 2005), the degradation of prohibitin is a likely mechanism by which Skp2B affects prohibitin levels.

To test this possibility, we first determined the half-life of prohibitin in MCF-7 and MCF-7-Skp2B cells. We observed that while the half-life of prohibitin is more than 1 h in MCF-7 cells, it was reduced to 30 min in MCF-7-Skp2B cells (Fig 2A). We next tested whether prohibitin is a substrate for ubiquitination and whether inhibiting the function of cullin 1 in MCF-7-Skp2B cells, by using a dominant-negative construct of cullin 1 or by inhibiting the proteasome, restores the stability of prohibitin. We observed that inhibition of cullin 1 increases the level of prohibitin 2.1-fold in cells overexpressing Skp2B (Fig 2B), whereas this increase was more modest in MCF-7 cells (supplementary Fig S1E online). Furthermore, we observed that inhibition of the proteasome in MCF-7-Skp2B cells, by using either LLnL or MG132, resulted in the accumulation of prohibitin fivefold and 7.9-fold, respectively (Fig 2C); this elevation was more modest in MCF-7 cells (supplementary Fig S1F online). In addition, we observed that upon transfection of Myc-tagged ubiquitin, immunoprecipitation of prohibitin and immunoblotting using a Myc antibody, polyubiquitinated forms of prohibitin are detected (Fig 2D). Furthermore, inhibition of Skp2B reduced the ubiquitination of prohibitin, whereas overexpression of Skp2B elevated markedly the ubiquitination of prohibitin (Fig 2D). Collectively, these results

Fig 2 | Skp2B promotes the ubiquitination of prohibitin. (A) MCF-7 and MCF-7-Skp2B cells were treated with 10 ng per ml of cycloheximide for the indicated time and the level of prohibitin at each time point was determined by western blot analysis. The numbers below the prohibitin blot represent the prohibitin/tubulin ratio. The western blot is representative of the result obtained in four replicates of this experiment ($P = 0.005$). (B) MCF-7-Skp2B cells were transfected with 5 μ g of Flag-tagged dominant-negative (dn) cullin 1 for 48 h. Levels of prohibitin were then determined by immunoblotting. The numbers below the prohibitin blot represent the prohibitin/tubulin ratio. (C) MCF-7-Skp2B cells were treated with LLnL (50 μ M) or MG132 (20 μ M) for 18 h and the level of prohibitin was determined by western blot analysis. The numbers below represent the prohibitin/tubulin ratio. (D) MCF-7 cells were transfected with or without 1 μ g of Myc-tagged Ub, 5 μ g of Skp2B or 10 nM of siRNA against Skp2B for 24 h and then LLnL was added (50 μ M). Ubiquitinated forms of prohibitin were detected using a Myc antibody. General ubiquitination of proteins in the total extracts by Myc-tagged Ub is shown in the lower panel and is used as a control for transfection efficiency of Myc-tagged Ub. CHX, cycloheximide; IgG, immunoglobulin; LLnL, leuciny-leuciny-norleuciny; siRNA, small interfering RNA; Ub, ubiquitin.

suggest that prohibitin is targeted for proteasomal degradation upon overexpression of Skp2B and the formation of an SCF^{Skp2B} ubiquitin ligase complex. As the SCF^{Skp2B} complex is cytoplasmic and prohibitin shuttles between the nucleus and the cytoplasm (Mishra *et al*, 2005), our data suggest that Skp2B prevents the translocation of prohibitin to the nucleus.

Prohibitin is required for p53 transcriptional activity

Prohibitin binds to and stimulates the transcriptional activity of p53 (Fusaro *et al*, 2003). To confirm the association between p53 and prohibitin, we used both MCF-7 and MCF-7-Skp2B cells for immunoprecipitation. As expected, p53 and prohibitin could be immunoprecipitated in MCF-7 cells but not in MCF-7-Skp2B cells (supplementary Fig S1G online). As the activity of p53 is reduced in cells overexpressing Skp2B (Fig 1B), we then tested whether p53 activity can be rescued by overexpressing prohibitin. The p53 reporter and a plasmid overexpressing prohibitin were transfected in MCF-7-Skp2B cells, which were then treated with 10 μ M of camptothecin. We observed that prohibitin rescued 50% of p53 activity (Fig 3A), a result that is consistent with the rapid turnover of prohibitin when Skp2B is overexpressed. To confirm the reduction in p53 reporter activity, we then tested the levels of endogenous p21, a target of p53, both at the protein and messenger RNA (mRNA) levels in MCF-7 and MCF-7-Skp2B cells after the addition of camptothecin. In both cell lines, the kinetics of p53 stabilization appeared similar, although in MCF-7 cells it resulted in the detection of endogenous p21 after 3 h (twofold), increasing further after 4 h (4.8-fold; Fig 3B), whereas in MCF-7-Skp2B cells the induction of p21 was delayed with an increase of 1.1-fold and twofold after 3 and 4 h, respectively (Fig 3B). The reduction in p21 transcription was more evident when the level of p21 mRNA was determined. We observed that although

the level of p21 mRNA increased 11-fold after treatment with 10 μ M of camptothecin for 12 h in MCF-7 cells, the level of p21 mRNA in Skp2B cells was only increased 2.5-fold (Fig 3C). The effect of Skp2B overexpression was confirmed by using two additional endogenous targets of p53, GADD45 (growth arrest and DNA damage) and PUMA (p53-upregulated modulator of apoptosis; supplementary Fig S1H,I online). Conversely, when endogenous Skp2B was inhibited by siRNA in MCF-7 cells, the activity of p53 was increased by 1.45-fold (Fig 3D), which is similar to that observed for the level of prohibitin (Fig 1E). To test whether the effect of Skp2B on p21 levels is dependent on p53, we performed two experiments: first, Skp2B was overexpressed in T47D cells, which express mutant p53 and the level of p21 was determined; second, Skp2B was transfected in the p53-null breast cancer cell line MDA-MB-157 and the activity of the p21 promoter-luciferase reporter determined. We observed that as expected, Skp2 overexpression led to a reduction in p21 levels in T47D cells but that of Skp2B has no effect (supplementary Fig S1J online). Furthermore, Skp2B overexpression did not affect the activity of p21 reporter in p53-null cells (supplementary Fig S1K). These results indicate that although the stabilization of p53 is not affected by Skp2B levels, in the absence of prohibitin, the recruitment of p53 to promoter is reduced (Fusaro *et al*, 2003) and as a result the transcriptional activity of p53 is attenuated.

Attenuated p53 activity in MMTV-Skp2B mice

We next analysed the level of prohibitin in wild-type and MMTV-Skp2B transgenic mice. The mammary glands of two wild-type and two MMTV-Skp2B transgenic mice were collected and analysed by western blotting. A reduction in prohibitin in the MMTV-Skp2B mice was observed compared with wild-type mice

Fig 3 | p53 activity is reduced in cells overexpressing Skp2B. (A) MCF-7-Skp2B cells were transfected with a p53 reporter (1 μ g) and a plasmid expressing prohibitin (5 μ g). At 24 h after transfection, cells were treated with 10 μ M of camptothecin for 18 h and the luciferase activity was determined. (B) MCF-7 and MCF-7-Skp2B cells were treated with 10 μ M of camptothecin and the levels of p53 and p21 were determined by western blot analysis. Tubulin was used as a loading control. The numbers below represent the p21/tubulin ratio. (C) MCF-7 and MCF-7-Skp2B cells were treated with 10 μ M of camptothecin for 18 h and the levels of endogenous p21 mRNA were determined by using 100 ng total RNA. (D) MCF-7 cells were transfected with 10 μ M of siRNA against Skp2B and 1 μ g of p53 reporter. After 24 h, the cells were treated with 10 μ M of camptothecin for a further 24 h and a luciferase assay was performed. CPT, camptothecin; IB, immunoblotting; mRNA, messenger RNA; siRNA, small interfering RNA.

(Fig 4A), supporting the observation made in MCF-7-Skp2B cells *in vitro*. We next aimed at addressing the consequence of p53 attenuation *in vivo* by using three separate approaches. First, as p53 is induced markedly during involution and results in the transcription of p21 (Jerry *et al*, 1998), to determine whether p53 signaling is defective *in vivo*, we analysed the levels of p53 and p21 during involution in wild-type and MMTV-Skp2B transgenic mice. As reported previously, p53 and p21 were absent during lactation but were induced markedly at day 1 of involution (Fig 4B) and their levels remained high thereafter (Fig 4B). In MMTV-Skp2B mice, however, p21 was undetectable at day 1 of involution and its level was weaker than that observed in wild-type mice at days 2 and 3 of involution (Fig 4B). At day 4, p21 in the mammary glands of MMTV-Skp2B mice reached the level observed at day 1 in wild-type mice (Fig 4B). This first experiment, therefore, suggests that in MMTV-Skp2B mice the p53 activity is attenuated during involution.

Multiple pregnancies lead to hyperplasia of the mammary glands of p53 heterozygote mice (Jerry *et al*, 1998). These observations are consistent with the finding that p53 is a mediator of the protective effect of pregnancy (Kuperwasser *et al*, 2000; Sivaraman *et al*, 2001; Lu *et al*, 2008) and that conversely, treatment with pregnancy-associated hormones leads to the formation of mammary tumours in p53-null mammary glands (Jerry *et al*, 2000). Therefore, as a second approach, we next

analysed the mammary glands of wild-type and MMTV-Skp2B mice after three pregnancies. We found hyperplasia of the mammary gland in MMTV-Skp2B mice (Fig 4C) but no effect in wild-type mice (Fig 4C). This result suggests that the protective effect of p53 during pregnancy is lost in MMTV-Skp2B mice.

Exposure of p53 heterozygous mice to the carcinogen 7,12-dimethyl-1, 2 benzantracene (DMBA) increases the rate of mammary tumour formation and reduces latency (Jerry *et al*, 1994; Storer *et al*, 2001). Therefore, as a third approach, wild-type and transgenic mice were exposed to DMBA. We observed that in MMTV-Skp2B transgenic mice the incidence of spontaneous mammary tumours was 10% after 52 weeks; however, the incidence increased to 47% within 17 weeks of DMBA treatment (supplementary Fig S1L online). Therefore, collectively these observations are consistent with the idea that reduction of prohibitin in MMTV-Skp2B mice does lead to a defect in p53 activity *in vivo*, which is reflected by a defect in p21 induction during involution, a loss of the protective effect of pregnancy and an increased sensitivity to DMBA.

DISCUSSION

Our data indicate that the overexpression of Skp2B promotes the ubiquitination and subsequent degradation of prohibitin and as a consequence results in an attenuated transcriptional activity of p53 both *in vitro* and *in vivo*. However, we cannot rule out that

Fig 4 | p53 function is attenuated in the mammary gland of MMTV-Skp2B mice. (A) Mammary glands from two wild-type and two MMTV-Skp2B transgenic mice were used for western blot analysis of prohibitin. Tubulin was used as a loading control. (B) Mammary glands from wild-type or MMTV-Skp2B transgenic mice were collected after 5 days of lactation (lac) or day (D)1, D2, D3 or D4 of involution and used for western blot analysis of p53 and p21. Tubulin was used as a loading control. (C) Hematoxylin/eosin staining of a mammary gland from a wild-type female and an MMTV-Skp2B female after 10 days of involution and three consecutive pregnancies. (D) Model of the effect of amplification of the *Skp2* locus on p53 activity. IB, immunoblotting; MMTV, mouse mammary tumour virus.

other, yet to be identified, substrates of Skp2B might also contribute to the attenuation of p53 activity. Skp2 overexpression also promotes the attenuation of p53 function by affecting p300 levels and activity (Kitagawa *et al*, 2008). We previously observed that in most cases, Skp2 and Skp2B are amplified in primary breast cancers (Radke *et al*, 2005). The model of the combined effect of Skp2 and Skp2B is presented in Fig 4D. These observations raise the possibility that even in absence of p53 mutations or mdm2 amplification, the activity of p53 might be attenuated owing to the amplification of the *Skp2* locus. Furthermore, as Skp2 promotes the degradation of the cyclin-dependent kinase inhibitor p27 (Carrano *et al*, 1999)—and although we found that Skp2B promotes the activity of the oestrogen receptor (Umanskaya *et al*, 2007)—the combined effect of Skp2 and Skp2B is predicted to be the acceleration of cellular proliferation in the context of an attenuated p53 checkpoint, and might indicate the worst prognosis associated with Skp2/2B overexpression. (Kitagawa *et al*, 2008). As Skp2 and Skp2B differ only at the C-terminal domain but are otherwise identical, molecules able to inhibit both isoforms are predicted to be the most promising.

METHODS

Cell lines, plasmids and transfections. The MCF-7 and MCF-7-Skp2B cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All transfections were performed with the Mirus reagent (TransIT-LT-1, Mirus, Madison, WI, USA).

Transfection of siRNAs. siRNA transfections were performed by using RNAiFect Reagent (Qiagen, Valencia, CA, USA). The sequences for the siRNAs inhibiting Skp2B expression were designed and used as described previously (Umanskaya *et al*, 2007): siRNA#1, 5'-AGCCAGUUUCCUCGCAUCUTT-3'; siRNA#2, 5'-UGUCAACAUCACACUGCGCTT-3'; siRNA#3, 5'-CCCAGAAUACACUGUGAAATT-3'.

Western blotting and immunoprecipitation. These methods were performed as described previously (Radke *et al*, 2005) with the following antibodies: rabbit anti-Prohibitin (Biolegend, San Diego, CA, USA), mouse prohibitin (Neomarkers, Fremont, CA, USA) for immunoprecipitation, mouse Flag (Sigma, Saint Louis, MO, USA), mouse anti-Skp2 (Zymed, San Francisco, CA, USA), mouse tubulin antibody (Sigma), mouse Myc antibody 9E10 to detect Myc-ubiquitin, and p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Luciferase assay. To measure the transcriptional activity of p53, MCF-7 and MCF-7-Skp2B cells were transfected with the reporter plasmid pGL3-E1bTATA and with a plasmid constitutively expressing the Renilla luciferase. Luciferase activity was measured by using a dual luciferase reporter assay system (Promega, Madison, WI, USA).

DMBA treatment. The carcinogen DMBA (Sigma) was dissolved in corn oil at a concentration of 10 mg per ml. The seven-week-old virgin female transgenic and wild-type mice were treated with four weekly doses of 100 μ l of a 10 mg per ml solution of DMBA by gavage.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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