NAD(P)H quinone oxidoreductase 1 inhibits the proteasomal degradation of the tumour suppressor p33^{ING1b}

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The tumour suppressor p33^{ING1b} (ING1b for inhibitor of growth family, member 1b) is important in cellular stress responses, including cell-cycle arrest, apoptosis, chromatin remodelling and DNA repair; however, its degradation pathway is still unknown. Recently, we showed that genotoxic stress induces p33^{ING1b} phosphorylation at Ser126, and abolishment of Ser126 phosphorylation markedly shortened its half-life. Therefore, we suggest that Ser126 phosphorylation modulates the interaction of p33^{ING1b} with its degradation machinery, stabilizing this protein. Combining the use of inhibitors of the main degradation pathways in the nucleus (proteasome and calpains), partial isolation of the proteasome complex, and *in vitro* interaction and degradation assays, we set out to determine the degradation mechanism of p33^{ING1b}. We found that p33^{ING1b} is degraded in the 20S proteasome and that NAD(P)H quinone oxidoreductase 1 (NQO1), an oxidoreductase previously shown to modulate the degradation of p53 in the 20S proteasome, inhibits the degradation of p33^{ING1b}. Furthermore, ultraviolet irradiation induces p33^{ING1b} phosphorylation at Ser126, which, in turn, facilitates its interaction with NQO1.

Keywords: tumour suppressor p33^{ING1b}; NQO1; protein degradation; proteasome; phosphorylation

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INTRODUCTION

Several studies have shown that p33^{ING1b} (ING1b for inhibitor of growth family, member 1b) is a tumour suppressor (Feng *et al*, 2002; Kataoka *et al*, 2003), and it has been reported that p33^{ING1b} is downregulated in several carcinomas. Overexpression of this protein inhibits cell growth, whereas its antisense expression promotes cell transformation (Garkavtsev *et al*, 1996). p33^{ING1b} is

important in cellular stress responses, including cell-cycle arrest, apoptosis, chromatin remodelling and DNA repair (Cheung & Li, 2001; Scott *et al*, 2001; Campos *et al*, 2004). This protein can also sensitize cells to stress agents such as etoposide and γ -irradiation in wild-type but not in p53-deficient cell lines (for a review see Campos *et al*, 2004). We found that p33^{ING1b} is induced by ultraviolet radiation in human keratinocytes and melanoma cells (Cheung *et al*, 2000, 2001). In addition, we showed that p33^{ING1b} markedly enhances nucleotide excision repair of ultraviolet-damaged DNA and promotes UVB-induced apoptosis in melanoma cells (Cheung *et al*, 2001; Cheung & Li, 2002). Recently, we discovered that p33^{ING1b} is phosphorylated at Ser126, and abolishment of Ser126 phosphorylation markedly shortened its half-life. Furthermore, phosphorylation at the Ser126 residue is important in the regulation of cyclin B1 expression and the proliferation of melanoma cells (Garate *et al*, 2007). These results indicate that the degradation of p33^{ING1b} is modulated by phosphorylation at the Ser126 residue; however, the exact degradation pathway of p33^{ING1b} is unclear. In this work, we examined the effect of specific protease inhibitors on the half-life of p33^{ING1b} and further confirm our results through the detection of this tumour suppressor in purified fractions of proteasome. Here, we provide evidence that p33^{ING1b} is degraded in the 20S proteasome. Our results indicate that the NAD(P)H quinone oxidoreductase 1 (NQO1) inhibits the degradation of p33^{ING1b} and that ultraviolet irradiation stabilizes p33^{ING1b} by inducing phosphorylation at Ser126, which enhances the p33^{ING1b} interaction with NQO1.

RESULTS AND DISCUSSION

To determine the degradation pathway of p33^{ING1b}, MMRU cells were treated for 16 h with different inhibitors. MG132 was the first choice, as p33^{ING1b} is localized in the nucleus where proteasome is the main degradation pathway. Calpain inhibitor I (Cil) was used as an alternative, as calpains are important proteases in the nucleus. Determination of p33^{ING1b} protein levels in nuclear extracts (Fig 1A) showed that MG132 caused an increase of $55 \pm 7\%$ in the expression of p33^{ING1b} compared with cells degraded in the absence of inhibitor, whereas Cil had no significant effect. To confirm the role of proteasome in the degradation of p33^{ING1b}, we

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Fig 1 | p33^{ING1b} is degraded in the proteasome. (A) p33^{ING1b} protein levels were determined by western blot in nuclear extracts of cells treated for 16 h with CHX or CHX plus MG132 or Cil. The density of the bands was determined and plotted as the relative fold compared with the untreated control. The bars represent the average $\pm$ s.d. from three independent experiments; * $P < 0.01$. (B) Effect of lactacystin on the half-life of p33^{ING1b}. p33^{ING1b} levels were determined in whole-cell extracts of cells treated with CHX (Control) or CHX plus lactacystin for different time points by western blot analysis. p53 levels were determined as a positive control of proteasomal degradation. The degradation rate of p33^{ING1b} was determined by densitometric analysis of bands as described in the Methods. Values are means $\pm$ s.d. CHX, cycloheximide; Cil, calpain inhibitor I; ING1b, inhibitor of growth family, member 1b.

determined the half-life of this protein by treating the cells with cycloheximide (CHX) in the presence or absence of lactacystin, an irreversible and specific inhibitor of proteasome. Consistent with our previous report (Garate *et al*, 2007), we estimated a half-life of 15.1 h for endogenous p33^{ING1b} (Fig 1B), and lactacystin notably extended its half-life by approximately threefold. We confirmed the accuracy of the method used in this work by determining the turnover rate of two proteins—checkpoint kinase 1 (Chk1) and cyclin-dependent kinase 1 (Cdk1)—of known half-life (supplementary Fig S1 online). Furthermore, through immunolocalization, we eliminated the possibility that MG132 might induce relocalization of p33^{ING1b} (supplementary Fig S2 online), suggesting that p33^{ING1b} is degraded in proteasomes.

As ubiquitination triggers most proteins to be degraded in the proteasome (Grossman *et al*, 2003; Zhang *et al*, 2005), we evaluated, by pull-down assays, whether p33^{ING1b} is ubiquitinated. Immunoprecipitation assays in Fig 2A show that there is a marked increase in the expression of both p53 (left panel) and p33^{ING1b} (centre panel) when the cells are incubated in the presence of MG132. Conversely, isolation of ubiquitinated proteins (as described in the supplementary information online) showed only a single band of ubiquitinated p33^{ING1b}, which did not increase in the presence of MG132 (right panel). Therefore, inhibition of proteasomal function did not affect the recovery of ubiquitinated p33^{ING1b} but, not surprisingly, it increased the recovery of ubiquitinated p53, a known substrate of ubiquitin-dependent and ubiquitin-independent proteasomal pathways (Asher & Shaul, 2005), where several bands were detected as a sign of polyubiquitination (Fig 2A, right panel). These results indicate that p33^{ING1b} is monoubiquitinated and degrades independently of ubiquitin. Furthermore, through ammonium sulphate fractionation of nuclear extracts (supplementary Fig S3 online), we found that p33^{ING1b} is present in the 20S proteasome-containing fraction (38–70% ammonium sulphate-soluble fraction) only when the cells were incubated in the presence of MG132, suggesting that p33^{ING1b} is degraded in the core of the proteasome. Further isolation of the 20S proteasome found that p33^{ING1b} co-fractionate with this complex in every step. To show conclusively that p33^{ING1b} is degraded in the 20S proteasome, we incubated affinity-isolated recombinant glutathione *S*-transferase (GST)-ING1b with purified mammalian 20S proteasome *in vitro* for different time points (Fig 2B, control). We found that 20S proteasome is able to degrade p33^{ING1b} but not GST alone (supplementary Fig S4 online). It has been shown previously that NQO1 modulates the degradation of proteins such as p53 and p73 (Asher *et al*, 2005a) or ornithine decarboxylase (Asher *et al*, 2005b) in the 20S proteasome. Therefore, we assessed the possibility that the degradation of p33^{ING1b} might be modulated by NQO1. NQO1 inhibited the degradation of p33^{ING1b} by approximately 40%, and the degradation was inhibited by more than 80% in the presence of its substrate (NADH). To determine whether NQO1 modulates the degradation of p33^{ING1b} *in situ*, NQO1 was knocked down. The results showed that knocking down NQO1 induced the degradation of p33^{ING1b} (Fig 2C). As NQO1 is stabilized by interacting with its partner proteins, we investigated, by co-immunoprecipitation, whether NQO1 interacts with p33^{ING1b} (Fig 2D). We were able to pull down p33^{ING1b} using antibodies specific for NQO1 in cells expressing control short hairpin RNA (shRNA) but not in cells expressing NQO1 shRNA, confirming the interaction p33^{ING1b} and NQO1.

It has been shown that phosphorylation targets various proteins for degradation, for example, Chk1 (Zhang *et al*, 2005) and p27 (Hasan *et al*, 2007). But other studies have clearly established that phosphorylation could also stabilize proteins such as p53 (Chehab *et al*, 1999) and p73 (Tsai & Yuan, 2003). In our recent work, we have shown that abolishment of Ser 126 phosphorylation shortens the half life of p33^{ING1b} (Garate *et al*, 2007). To investigate whether p33^{ING1b} phosphorylation at Ser 126 determines the degradation pathway of this protein, we assessed the effect of MG132 and Cil on the degradation of ectopic wild-type or unphosphorylatable S126A mutant p33^{ING1b}. Consistent with the effect shown on the degradation of endogenous p33^{ING1b} (Fig 1A),

Fig 2 | p33^{ING1b} is degraded in the 20S proteasome complex. (A) Nuclear extracts from cells treated for 16 h in the presence (+) or absence (-) of MG132 were immunoprecipitated (IP) with p53 and p33^{ING1b} antibodies. Nuclear extracts expressing polyhistidine-tagged ubiquitin (His-Ub) were pulled down (PD) with Ni-NTA agarose beads and probed against p33^{ING1b}. p53 was used as a positive control of proteasomal degradation. β -Actin was detected as an input control in aliquots of nuclear extracts before the assay. (B) NQO1 protects p33^{ING1b} against degradation in the 20S proteasome *in vitro*. Isolated GST-ING1b was incubated with purified 20S proteasome at 37 °C for different time points in the absence or presence of isolated NQO1 with or without 1 mM NADH. Samples were blotted and probed with antibodies against p33^{ING1b} and NQO1. BSA (added to stabilize the enzymes) was detected as a negative control of degradation in the 20S proteasome. The degradation rate of p33^{ING1b} was determined by densitometric analysis of the bands. Values are means $\pm$ s.d. (C,D) NQO1 modulates the degradation of p33^{ING1b} *in situ*. (C) Nuclear extracts from cells transfected with a plasmid encoding either NQO1-specific shRNA (pSup-NQO1) or control shRNA (pSup-Ctrl) and treated with MG132 and CHX for 16 h were blotted and probed with a p33^{ING1b} antibody. The blots were also probed for NQO1 to ensure successful knockdown. p53 was used as a positive control of proteasomal degradation. (D) Nuclear extracts from cells transfected with either pSup-NQO1 or pSup-Ctrl were immunoprecipitated with an NQO1 antibody, blotted and probed for p33^{ING1b} or NQO1. β -Actin was detected as an input control in aliquots of nuclear extracts before immunoprecipitation. BSA, bovine serum albumin; CHX, cycloheximide; GST, glutathione S-transferase; ING1b, inhibitor of growth family, member 1b; NQO1, NAD(P)H quinone oxidoreductase 1; shRNA, short hairpin RNA.

MG132 inhibited the degradation of both wild-type and S126A mutant p33^{ING1b} (Fig 3A), causing an increase of $65 \pm 6\%$ and $117 \pm 7\%$, respectively, in their expression compared with untreated cells. This suggests that p33^{ING1b} is degraded in the proteasome complex independently of Ser126 phosphorylation. Nevertheless, the fact that p33^{ING1b} has a discrete signal for chaperone-mediated autophagy (CMA), $_{45}\text{QELK}_{49}$, persuaded us to assess the possibility that unphosphorylatable p33^{ING1b} could be misfolded and degraded by autophagy. Our results show that neither form of p33^{ING1b} is degraded by autophagy, as it is not possible to detect them in isolated lysosomes even in the presence of inhibitor (supplementary Fig S5 online). Therefore, we assessed whether phosphorylation at Ser126 could affect the interaction of p33^{ING1b} and NQO1. Through immunoprecipitation of this oxidoreductase from cells expressing wild-type, S126A or S126E (phosphomimetic) mutant p33^{ING1b}, we found that the phosphomimetic form co-immunoprecipitated to a larger extent with NQO1 than the partly phosphorylated wild-type p33^{ING1b}, whereas the unphosphorylatable S126A mutant did not co-precipitate with NQO1 (Fig 3B). The interaction of phosphorylated p33^{ING1b} and NQO1 was confirmed by an independent pull-down assay (supplementary Fig S6 online). We found that NQO1 present in the nuclear extracts of MMRU cells binds more readily to phosphorylated GST-ING1b than to the unphosphorylated form, whereas GST is not able to bind to this oxidoreductase. To determine whether p33^{ING1b} interacts directly with NQO1, we incubated GST-ING1b and isolated NQO1 *in vitro* in the absence or presence of NADH (Fig 3C). We found that NQO1 binds more readily to GST-ING1b in the presence of its substrate (NADH), but

NQO1 does not bind to GST either in the presence or absence of NADH. To determine whether NQO1 differentially modulates the degradation of phosphorylatable and non-phosphorylatable p33^{ING1b} *in situ*, we knocked down this enzyme. As shown in Fig 3D, knocking down NQO1 accelerated the degradation of ectopic wild-type p33^{ING1b}, but not the S126A mutant compared with control cells, suggesting that phosphorylatable p33^{ING1b} is stabilized preferentially by this oxidoreductase. Consistent with our previous results (Garate et al, 2007), the S126A mutant is degraded faster than the wild-type p33^{ING1b}. The addition of MG132 ameliorates the degradation of both forms of p33^{ING1b}, confirming that they share the same degradation pathway. Furthermore, these results confirm that NQO1 interacts preferentially with phosphorylated p33^{ING1b}.

We have shown previously that ultraviolet irradiation induces p33^{ING1b} phosphorylation at Ser126 (Garate et al, 2007). To explain the role of ultraviolet irradiation in p33^{ING1b} degradation, we irradiated cells with different dosages of UVB and determined its effect on p33^{ING1b} expression, phosphorylation and its interaction with NQO1. We found that ultraviolet irradiation induced both the expression and phosphorylation of p33^{ING1b},

Fig 3 | NQO1 preferentially binds to phosphorylated p33^{ING1b}. (A) Phosphorylation at Ser 126 does not alter the degradation pathway of p33^{ING1b}. Nuclear extracts obtained from cells transfected with pCI-NeoING1b (WT) or pCI-NeoS126A (S126A), and treated for 16 h with CHX alone (control) or combined with MG132 or Cil, were blotted and probed for p33^{ING1b} or p53. The intensity of p33^{ING1b} signal in each band was quantified and plotted as the relative fold compared with that of untreated cells. Values represent average $\pm$ s.d. from three independent experiments. * $P < 0.01$; ** $P < 0.001$. (B) NQO1 preferentially binds to p33^{ING1b} phosphorylated at Ser 126. Aliquots of 50 μ g of nuclear extracts prepared from cells expressing WT, S126A or S126E mutant p33^{ING1b} were blotted. p33^{ING1b} and NQO1 expression and phosphorylation at Ser 126 were determined by western blot using specific antibodies (left panel). Aliquots of 250 μ g nuclear extracts were immunoprecipitated (IP) with an NQO1 antibody and the immunocomplexes were analysed by western blotting for p33^{ING1b}, Ser 126 phosphorylation or NQO1 (right panel). (C) GST or GST-ING1b was incubated with isolated NQO1 in the absence or presence of NADH. The complexes isolated by GST pull-down were blotted, and NQO1 and GST-ING1b levels were determined by western blot and that of GST by Ponceau S staining. (D) Nuclear extracts from cells co-transfected with a plasmid encoding NQO1-specific shRNA (pSup-NQO1) or control shRNA (pSup-Ctrl) and pCI-NeoING1b (WT) or pCI-NeoS126A, and treated with MG132 and CHX for 16 h were blotted and probed for p33^{ING1b} and NQO1. p53 was used as a positive control of proteasomal degradation. Cil, calpain inhibitor I; CHX, cycloheximide; GST, glutathione S-transferase; ING1b, inhibitor of growth family, member 1b; NQO1, NAD(P)H quinone oxidoreductase 1; shRNA, short hairpin RNA; WT, wild type.

reaching a maximum at a dose of 10 mJ/cm² (Fig 4A, left panel). By immunoprecipitation of NQO1 from control or ultraviolet-irradiated cells, we found that ultraviolet irradiation enhanced the interaction of p33^{ING1b} with NQO1 (Fig 4A, right panel). The induction of p33^{ING1b} phosphorylation also correlates with markedly increased stabilization of this protein. A half-life of 15.4 h is estimated for p33^{ING1b} under control conditions, whereas it is extrapolated to be 57.6 h after ultraviolet irradiation (Fig 4B).

CONCLUSION

Our data provide evidence that under normal conditions poorly phosphorylated p33^{ING1b} is degraded in the 20S proteasome (Fig 5A). This pathway is mediated by NQO1, which interacts preferentially with phosphorylated p33^{ING1b} and partly inhibits its degradation in the 20S proteasome. Ultraviolet irradiation induces p33^{ING1b} phosphorylation at Ser 126, inducing further stabilization of this protein through interaction with NQO1 (Fig 5B).

METHODS

Cell culture, inhibitors and treatments. The human melanoma cell line MMRU (a kind gift from Dr R. Byers, Boston University School of Medicine) was maintained as described previously (Garate *et al*, 2007). Transfection of different DNA vectors was

performed using Effectene reagent (Qiagen Inc., Mississauga, ON, Canada), according to the manufacturer's protocol, followed by a round of selection in media containing 1 mg/ml G418. Under these conditions, a transfection efficiency of more than 95% was achieved.

The different inhibitors and DNA vectors used in this work, as well as their respective concentrations, are summarized in the supplementary information online.

Western blot analysis. Nuclear pellets were obtained as described previously (Garate *et al*, 2007) and proteins were solubilized in nuclear extract buffer (20 mM Hepes, pH 7.9, 0.35 M NaCl, 1 mM EDTA, 20% glycerol, 1 mM DTT) plus protease and phosphatase inhibitors. The concentration of proteins was determined by using the DC Protein Assay (Bio-Rad, Hercules, CA, USA) and western blot analysis was performed using the Odyssey Infrared Imaging System as described previously (Razidlo *et al*, 2004). The intensity of the signal of interest was corrected by detecting β-actin as the input control. Immunoprecipitation was performed as described previously (Doyon *et al*, 2004).

Determination of p33^{ING1b} half-life. MMRU cells treated with CHX were collected at different time points and whole-cell proteins were extracted as described previously (Wang & Li, 2006).

Fig 4 | UVB irradiation induces binding of p33^{ING1b} to NQO1 through phosphorylation at Ser 126. (A) p33^{ING1b} expression and phosphorylation at Ser 126 were determined in nuclear extracts of cells irradiated with different dosages of UVB (mJ/cm²) and treated with CHX by western blot using specific antibodies (left panel). Aliquots of two nuclear extracts (250 µg each) were immunoprecipitated (IP) with an NQO1 antibody and the levels of p33^{ING1b} or NQO1 or phosphorylation at Ser 126 in the immunocomplexes were assessed (right panel). (B) Effect of UVB irradiation on the half life of p33^{ING1b}. MMRU cells were subjected UVB irradiation (10 mJ/cm²) and treated with CHX. p33^{ING1b} protein levels were determined in nuclear extracts from these cells by western blot. (C) The degradation rate of p33^{ING1b} was determined by densitometric analysis of the bands. Values are means ± s.d. CHX, cycloheximide; ING1b, inhibitor of growth family, member 1b; NQO1, NAD(P)H quinone oxidoreductase 1.

for western blotting using a p33^{ING1b} antibody. The half-life was estimated from the band density as described previously (Cuervo et al, 1998).

In vitro degradation of p33^{ING1b} in the 20S proteasome. Recombinant GST-ING1b and NQO1 were isolated as described in the supplementary information online. Purified mammalian 20S proteasomes were purchased from Sigma-Aldrich (Oakville, ON, Canada).

A 250 ng portion of isolated GST-ING1b was mixed with similar amounts of bovine serum albumin, 100 ng of 20S proteasomes, 50 ng of NQO1 and 1 mM NADH in different combinations in degradation buffer (100 mmol/l Tris-HCl (pH 7.5), containing 150 mmol/l NaCl, 5 mmol/l MgCl₂ and 2 mmol/l dithiothreitol) and incubated for different time points at 37 °C. All samples were boiled in Laemmli denaturation buffer and analysed by western blot. **Statistical analysis.** Student's *t*-test was used to perform statistical analyses. A *P*-value <0.05 was considered significant.

Fig 5 | Proposed model for the effect of UVB irradiation on the mechanism of p33^{ING1b} degradation. NQO1 preferentially binds to p33^{ING1b} phosphorylated at Ser 126. (A) Under the conditions of non-ultraviolet stress, p33^{ING1b} shows a low degree of phosphorylation at Ser 126 and is degraded readily in the 20S proteasome complex in the nucleus. (B) On UVB irradiation, p33^{ING1b} is efficiently phosphorylated at Ser 126, inducing its binding to NQO1, which, in turn, stabilizes p33^{ING1b}. ING1b, inhibitor of growth family, member 1b; NQO1, NAD(P)H quinone oxidoreductase 1.

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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