Dissection of the functional interaction between p53 and the embryonic proto-oncoprotein PAX3

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Introduction

The p53 tumour suppressor is a nodal point for the response to a range of cellular stresses [1]. It suppresses cellular replication and survival primarily by promoting the transcription of specific target genes, including *WAF1*, which encodes the cyclin-dependent kinase inhibitor $p21^{WAF1}$, and BH3 family pro-apoptotic genes *BAX*, *PUMA* and *NOXA* [1]. p53 also activates the transcription of the gene encoding its negative regulatory partner, HDM2 (Mdm2 in mice) [1,2], ensuring p53 activity is maintained at low levels in proliferating cells. The importance of correct control of p53 is clearly demonstrated by the p53-dependent embryonic lethality observed in $Mdm2^{-/-}$ mice [3,4] and the ability of pharmacological HDM2 antagonists to inhibit the proliferation of wild-type p53-expressing cancer cells [5].

Pax proteins are a family of developmentally regulated transcription factors with roles in proliferation, differentiation and survival [6]. Mouse embryos homozygous for an inactivating mutation in *Pax3* (*Splotch* mice) die in mid-gestation with a variety of neural tube defects and cardiac abnormalities [7]. The neuroepithelial tissue of these embryos exhibit a post-transcriptional increase in p53 protein abundance, and p53-dependent apoptosis with associated neural tube defects [8]. *Pax3* is down-regulated in later development and its expression in the adult is restricted to a small number of highly specialised stem cells such as melanocytes in the bulge region of hair follicles [9]. *PAX3* is, however, frequently expressed in human cancers derived from cells of neural crest origin, notably neuroblastoma and melanoma [6], and inhibition of *PAX3* expression in melanoma cells results in p53 protein induction and apoptosis [10]. These findings indicate that a primary role of *PAX3* in the developing neural crest and

potentially also certain human tumours, involves the suppression of p53-dependent apoptotic pathways.

In contrast to HDM2, which is well documented as both inhibiting the interaction of p53 with transcriptional co-activators, and targeting p53 for ubiquitination, nuclear export and degradation [1]; the mechanism whereby PAX3 regulates p53 function is unknown. Here we describe the results of a series of experiments to dissect the molecular basis for the functional interaction between these two proteins.

2. Materials and Methods

2.1 Cell culture, plasmids, transfections, mRNA and reporter gene assays

H1299 and NIH3T3 cells were grown in RPMI 1640 (Invitrogen), and DMEM (Invitrogen) respectively, supplemented with 10% foetal bovine serum (Autogen Bioclear). The Hdm2luc03 and Hdm2luc13 luciferase reporter vectors contain 165 b.p. (-132 to +33) and 616 b.p. (-583 to +33) of the *HDM2*-P2 promoter region respectively [11]. Hdm2luc17 is a derivative of Hdm2luc13 with two independent 2 b.p. substitutions in its two p53-response elements. Baxluc contains a 369 b.p. fragment of the *BAX* promoter in pGL3Basic [12]. Expression vectors for p53 (pC53SN3), and HDM2 (pCMVmdm2) as well as the *WAF1* promoter reporter (p21luc) were kind gifts from Professor Bert Vogelstein. pCDNA3p53F19A has been described [13]. pJ7PAX3 contains the entire human *PAX3* coding sequence (splice form *PAX3*c [14]) in pJ7ω. Site–directed mutagenesis of pJ7PAX3 used the following primers (forward primers only are

shown): Stop281 (5'- CTGGAGCCAATTAACTGATGGCTTTCAACC-3'), SD42 (5'-CGAGTCAACCAGCTCCGAGGAGTATTTATC-3'), WS265 (5'-GAGGCCCGAGTGCAGTTCTGGTTTAGCAAC-3'), N47H (5'-GGAGGAGTATTTATCCACGGCAGGCCTCTGCC-3'). All cell transfections used Lipofectamine 2000 reagent (Invitrogen) and total amounts of transfected plasmid were equalised using empty expression vector. Reporter assays were performed 48 h after transfection of cells in 96 well plates using a Dual-Glo^{TM} luciferase assay (Promega), with normalisation to *Renilla* luciferase expressed from co-transfected pRLSV40 (Promega) to produce relative luciferase units (RLU). Data is expressed as ± S.E.M., or S.D. where n=2. One-way ANOVA and Tukey HSD post-hoc testing with SPSS software were used for the statistical analysis of the effect of PAX3 in reporter assays. Asterisks indicate significance of the difference from the relevant no PAX3 control; *$p<0.05$, **$p\leq0.001$. Reverse transcriptase(RT)-quantitative (q)PCR was performed as described previously [15].

2.2 Protein analysis

Cells were washed with phosphate-buffered saline, pelleted by centrifugation at 1000 *g*, snap frozen and stored at -80 °C. Pellets were lysed for 15 min at 4 °C in denaturing urea buffer (7 M urea, 0.1 M dithiothreitol, 0.05% Triton X-100, 25 mM NaCl, 20 mM HEPES pH 7.6) then clarified by centrifugation at 13000 *g* for 10 min at 4 ºC. Protein concentrations were determined by the method of Bradford (Bio-Rad). Immunoblotting was performed by standard procedures and membranes were probed for PAX3 (Rabbit polyclonal, Active Motif), p53 (pAb421 or DO-1, Serotec) or EGFP (ab290, Abcam). DO-1 was used to detect p53 protein unless indicated otherwise. Bands were visualised by chemiluminescence (Supersignal, Pierce) using a Fluor-S

MAX system (Bio-Rad), and quantified using Quantity One software (Bio-Rad). Quantification of the blots is expressed relative to EGFP.

3. Results

3.1 PAX3 inhibits p53-induced transcription

Using a transient over-expression system in which the levels and activities of the two proteins could be readily manipulated and analysed, we first investigated the effect of PAX3 on p53-dependent transcription from the promoter of the *BAX* gene in cells which express low (NIH3T3, Fig. 1A) or no (H1299, Fig. 1B) endogenous p53 protein. In both cell lines, p53 caused a dose-dependent activation of the *BAX* promoter. This activation, but not basal promoter activity, was significantly inhibited by PAX3 ($p<0.001$ in NIH3T3, at 2.5 ng p53 plasmid plus 100 ng PAX3 plasmid and $p<0.001$ in H1299, at 5 ng p53 plasmid plus 100 ng PAX3 plasmid). We then extended this analysis to the *HDM2* promoter, which is activated by much lower levels of p53 than the *BAX* promoter, and is therefore a better model promoter, as it reduces the possibility of p53-induced cell death influencing the experimental results. PAX3 caused significant inhibition of p53-dependent *HDM2* promoter activity in H1299 reporter assays (Fig. 1C) ($p<0.001$ at 0.0625 ng p53 plasmid plus 100 ng PAX3 plasmid). Importantly, PAX3 also suppressed p53-dependent expression of the endogenous *HDM2-P2* transcript (Fig. 1D). Fig. 1E confirms that repression of *HDM2* promoter activity by PAX3 is dependent upon the presence of functional p53-response elements in the promoter (compare Hdm2luc13, with Hdm2luc17, in which the p53-response elements are mutated). Finally, we examined the ability of PAX3 to repress p53-

dependent transcription from the *WAF1* promoter (p21luc, Fig. 1F). In contrast to the *HDM2-P2* and *BAX* promoters, PAX3 over-expression caused a modest, but reproducible, up-regulation of *WAF1* reporter activity in both the absence and presence of transfected p53. This may indicate that the ability of PAX3 to regulate p53-dependent transcription of target genes can be promoter-dependent.

Suppression of p53 function by PAX3 involves regulation of p53 protein abundance, but is independent of p53 binding to HDM2

The activity of p53 as a transcription factor can be regulated at multiple levels including sequence-specific DNA-binding, interaction with co-activators, sub-cellular localisation and overall protein levels. In Fig. 2A we show that co-expression of PAX3 with p53 in the H1299 cell line causes a striking reduction in p53 protein levels, for example to 17% and 26% of no PAX3 controls in 0.6 ng and 1.2 ng p53-expression-vector lanes respectively. Fig. 2B demonstrates that PAX3 over-expression increases the rate of turnover of p53 protein in H1299 cells. These findings are consistent with the increase in p53 protein levels in PAX3 compromised melanoma cells [10], and murine embryos [8], though the magnitude of the effect was greater, presumably as a consequence of the over-expression system used.

We next investigated the requirement for HDM2 in PAX3-mediated repression of p53 abundance and function. The phenylalanine residue at position 19 of the amino terminus of p53 is critical for the primary interaction between p53 and the N-terminus of HDM2. This interaction causes a conformational shift in HDM2 that promotes a secondary interaction between the two molecules

that is required for p53 ubiquitination. Consequently, the F19A mutant of p53 is not targeted for degradation by HDM2 *in vivo* unless a p53 N-terminus mimic is present to activate HDM2 in *trans* [13,16,17]. Firstly, we established that it was necessary to transfect 0.5 ng of the p53F19A expression plasmid to induce a comparable level of p53-dependent transcription of Hdm2luc03 reporter plasmid as obtained in Fig. 1 using the wild-type p53 expression vector (not shown). Fig. 2C shows (insert) that levels of the p53F19A protein are decreased by PAX3 over-expression. PAX3 also caused a 6-fold reduction in the activation of a p53-dependent reporter construct by 0.5 ng of p53F19A (Fig. 2C). Therefore, neither the reduction of p53 protein level, nor the inhibition of p53-dependent transcription by PAX3, requires binding of p53 through its N-terminal domain to its primary cellular inhibitor, HDM2.

PAX3 must retain full integrity as a transcription factor to inhibit p53 function

PAX3 translated from the best characterised *PAX3c* mRNA splice form is a 479 amino acid protein. It has a modular structure with two distinct sequence-specific DNA-binding domains (paired domain and homeodomain), an N-terminal transcription inhibitory domain and a C-terminal transcription activation domain (Fig. 3A) [14,18]. To investigate the structural elements of PAX3 required for inhibition of p53 function, we created a series of mutant PAX3 expression constructs. The truncation mutant Stop 281 was designed to lack the transactivation domain, SD42 corresponds to an inactivating point mutation in the paired domain found in the mouse mutant *Splotch-delayed* [19]. This mutant affects PAX3 transcriptional activity but has a less severe phenotype than the *Splotch* mutant used by Pani *et al* [8]. WS265 encodes PAX3 with an inactivating point mutation in the homeodomain that is associated with Waardenburg syndrome in humans [20,21]. Expression of each of the mutant proteins was confirmed in H1299 cells (Fig.

3B). All these mutations also markedly, though not entirely, reduced the ability of PAX3 to diminish p53 protein abundance (Fig. 3B). The mutants were then tested for repression of p53-dependent activity of the *BAX* (Fig. 3C) and *HDM2* (Fig. 3D) promoters. Whilst, as before, wild-type PAX3 repressed p53-dependent transcription from both promoters ($p<0.001$ *BAX*, $p<0.001$ *HDM2*), none of the mutant PAX3 proteins had this effect.

Biochemical and mutagenesis studies have shown that the two DNA-binding domains of PAX3 are functionally interdependent [21,22]. Indeed, the SD42 mutation results in loss of DNA-binding by both paired- and homeo-domains [22]. We therefore generated a further PAX3 mutant N47H (Fig. 3E), that has been shown to abrogate DNA-binding by the paired domain but increase homeodomain DNA-binding activity [21]. N47H expressed in H1299 cells (Fig. 3E) was unable to repress p53-dependent transcription (Fig. 3F). Therefore, the ability of PAX3 to repress p53-dependent transcription is dependent upon the ability of both its paired- and homeo-domains to bind DNA (shown by N47H and WS265 mutants respectively), as well as the presence of an intact transcriptional activation domain, as demonstrated by the Stop 281 mutant.

Discussion

Both Pani *et al* [8] and He *et al* [10] demonstrated that loss of PAX3 expression can result in a post-transcriptional increase in p53 protein abundance, as well as apoptosis which, in the case of the developing neural tube, is p53 dependent [8]. Here we have shown that this effect of PAX3 on p53 protein abundance, and more specifically its rate of degradation, can be recapitulated in the H1299 over-expression system, confirming the existence of a functional interaction between

these two proteins. The most widely recognised mechanisms for the regulation of p53 protein degradation involves an interaction between p53 and its ubiquitin E3-ligase HDM2, which results in the ubiquitination and proteosome-mediated degradation of p53 [1,23]. It was of interest, therefore, to discover that a well-characterised, HDM2-binding defective mutant of p53, F19A, was still down-regulated by PAX3. Therefore, whilst a role of HDM2 that is independent of its binding to its primary interaction site on p53 has not been formally excluded, these data suggests the involvement of an alternative p53 ubiquitin E3-ligase such as COP1, [24] or an ubiquitin-proteosome independent pathway.

P53 protein stability and its activity as a transcription factor may be independently regulated [1], and previous reports have not investigated the effects of PAX3 loss on the transcriptional targets of p53. Here we have shown that PAX3 does indeed inhibit the p53-dependent transcription from at least two p53-dependent genes, including that encoding the pro-apoptotic BAX protein. Intriguingly, the effects of PAX3 exhibited a degree of specificity to different p53-responsive promoters; specifically whilst *HDM2-P2* and *BAX* promoters were suppressed, the *WAF1* promoter was not. Further work will be required to establish whether this results in a differential regulation of p53-depenent apoptosis, compared to cell-cycle arrest, by PAX3.

We have also clearly demonstrated that mutations in either of the two sequence-specific DNA-binding domains in PAX3, or deletion of its C-terminal transactivation domain, abrogate its ability to inhibit p53 activity. These PAX3 mutants do retain a partial ability to reduce p53 protein abundance, suggesting that, as is the case for HDM2, the effect of PAX3 on cellular p53 activity may not be solely through the regulation of p53 abundance. Our analysis of the effects of PAX3 mutations on p53 function are consistent with a recent analysis of the effects of over-

expressing *PAX3* splice forms on the response of melanocytes to genotoxic chemotherapy, in that *PAX3c* and *PAX3d*, which are expressed in melanomas, provided protection from etoposide-induced apoptosis, whereas *PAX3a* and *PAX3b,* which produce truncated proteins lacking the transactivation and homeodomains, did not [25]. Our interpretation of these data is either that PAX3 is a transcriptional activator of a gene, or genes, that regulate p53 function, and that only PAX3 proteins with functional paired- and homeo-DNA-binding domains are capable of activating the transcription of these genes, or that PAX3 and p53 proteins interact directly, but only fully functional PAX3 is able to influence p53 activity. Future work will be required to distinguish between these two possibilities, and fully determine the molecular basis for the functional interaction between these two key proteins.

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Figure Legends

Fig. 1. Effects of PAX3 on p53-dependent transcription**.** NIH3T3 (A) H1299 cells (B) were transfected with 100 ng of Baxluc or pGL3Basic, 20 ng pRLSV40 and the indicated amount of p53 and PAX3 vectors, and luciferase reporter assays performed (n=4). In (C), (E) and (F) similar experiments were conducted in H1299 cells using Hdm2luc03 (n=8), Hdm2luc13 and 17 (n=2) and p21luc (*WAF1* promoter) (n=4) reporters respectively. In (D) pGL3Basic was used instead of a reporter vector and RNA was extracted 48 h post transfection. The abundance of endogenous *HDM2-P2* mRNA transcripts was determined by RT-qPCR. Data is normalised to

GAPDH, and is mean ± S.D. for duplicate qPCR assays, and is representative of two independent experiments (Transfections were in 60 mm dishes. For ease of comparison, the amounts of plasmids shown are the equivalents by surface area of a 96 well plate transfection.).

Fig. 2. Effect of PAX3 on p53 protein abundance in H1299 cells**.** (A) Cells were transfected in 60 mm dishes with 5 ng EGFPN1, 1.8 µg pGL3Basic, p53 plasmid and 1.8 µg of pJ7PAX3 (+) or pJ7 empty vector (-) and analysed 48 h later. (B) H1299 cells were transfected in 60 mm dishes with 1.125 ng p53 plasmid, 1.8 µg pGL3 Basic, 5 ng EGFPN1 and 0.9 µg pJ7PAX3 or empty vector as indicated. At 48 h post transfection, 100 µg/ml cycloheximide (CHX) was added and cells harvested for analysis at the times indicated. Representative of three independent experiments. Note that for the p53 blot the + PAX3 lanes are a longer exposure than the No PAX3 lanes to facilitate comparison of p53 turnover. (C) For luciferase reporter assays, cells were transfected with 100 ng of Hdm2luc03 or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53F19A and PAX3 expression plasmids. (n=4). p53F19A protein was detected by western blotting of lysates from scaled up transfections, using antibody pAb421.

Fig. 3. Dissection of the PAX3 functional domains required to inhibit p53-dependent transcription**.** (A) PAX3 protein structure, and a description of the mutants used in the study. ID = inhibitory domain, PD = paired domain, OM = octapeptide motif, HD = homeodomain, TA = transactivation domain. (B) H1299 cells were transfected in 60 mm dishes with 5 ng EGFPN1, 1.8 µg pGL3Basic, 0.5 ng p53 plasmid and 0.9 µg of pJ7 empty vector or the appropriate PAX3 mutant plasmid as indicated. Transfected cells were analysed by western blotting 48 h later. (C & D) PAX3 mutants were tested for repression of p53-dependent transcription in a reporter assay in H1299 cells in a 96 well format. Cells were transfected with 20 ng pRLSV40, 100 ng pJ7

empty vector or the indicated PAX3 mutant and 100 ng reporter vector ((C) Hdm2luc03) or ((D) Baxluc). Open bars = 0 ng p53 plasmid, closed bars = 0.0625 ng p53 plasmid (C) or 2.5 ng p53 plasmid (D). (n=8). (E) The PAX3 mutant N47H was generated, and expression confirmed as in (A). (F) N47H was tested for repression of p53-dependent *HDM2* promoter activity as in (C). (n=2).

Fig 1

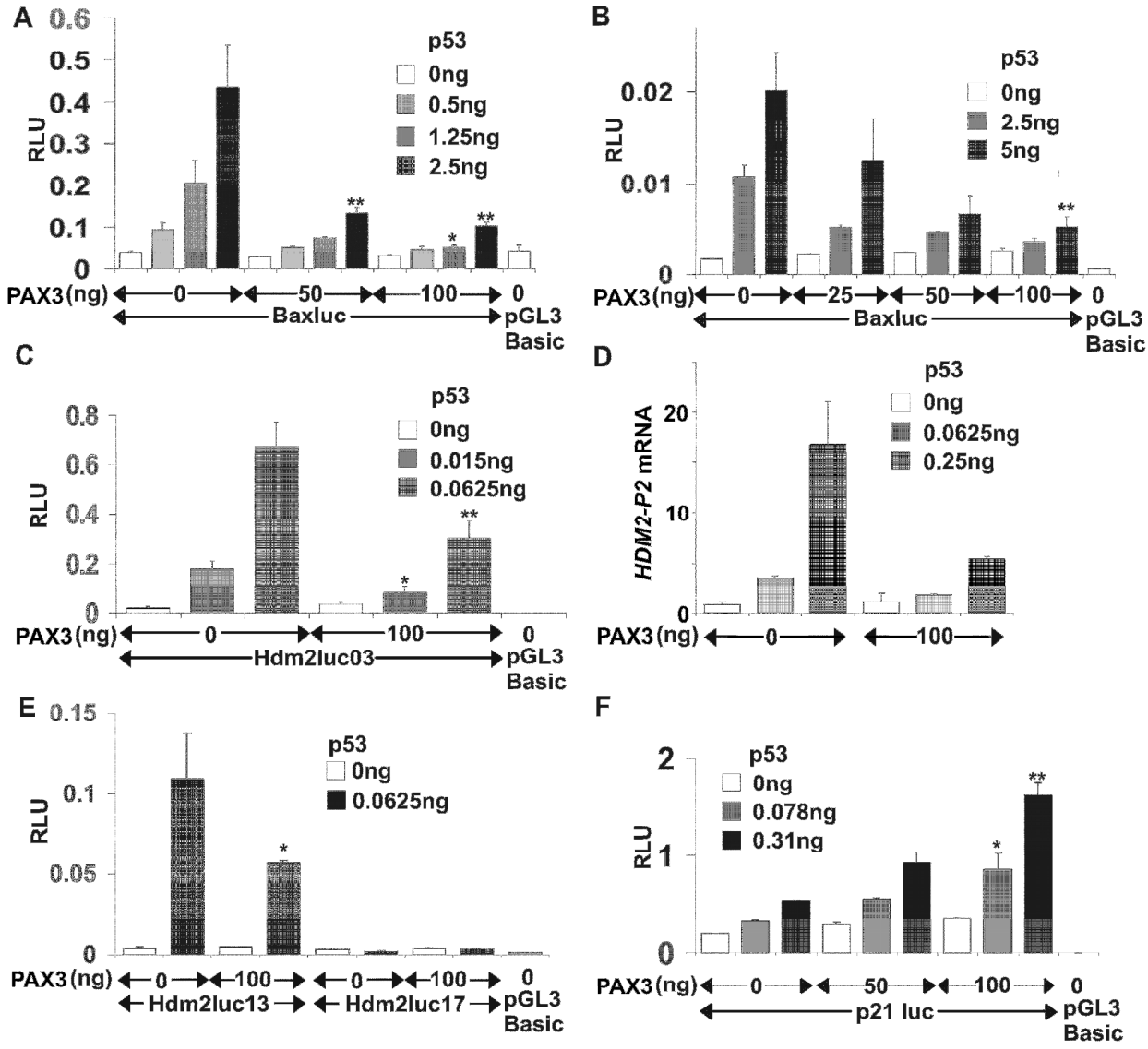
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Fig. 2

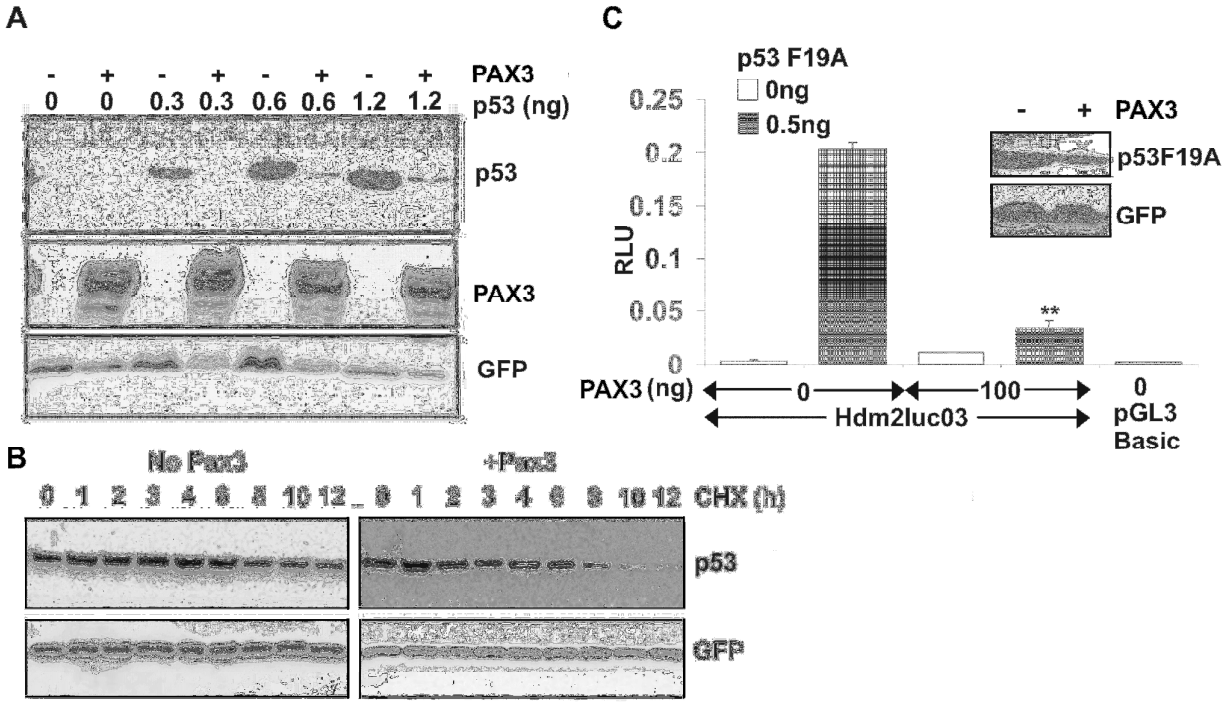
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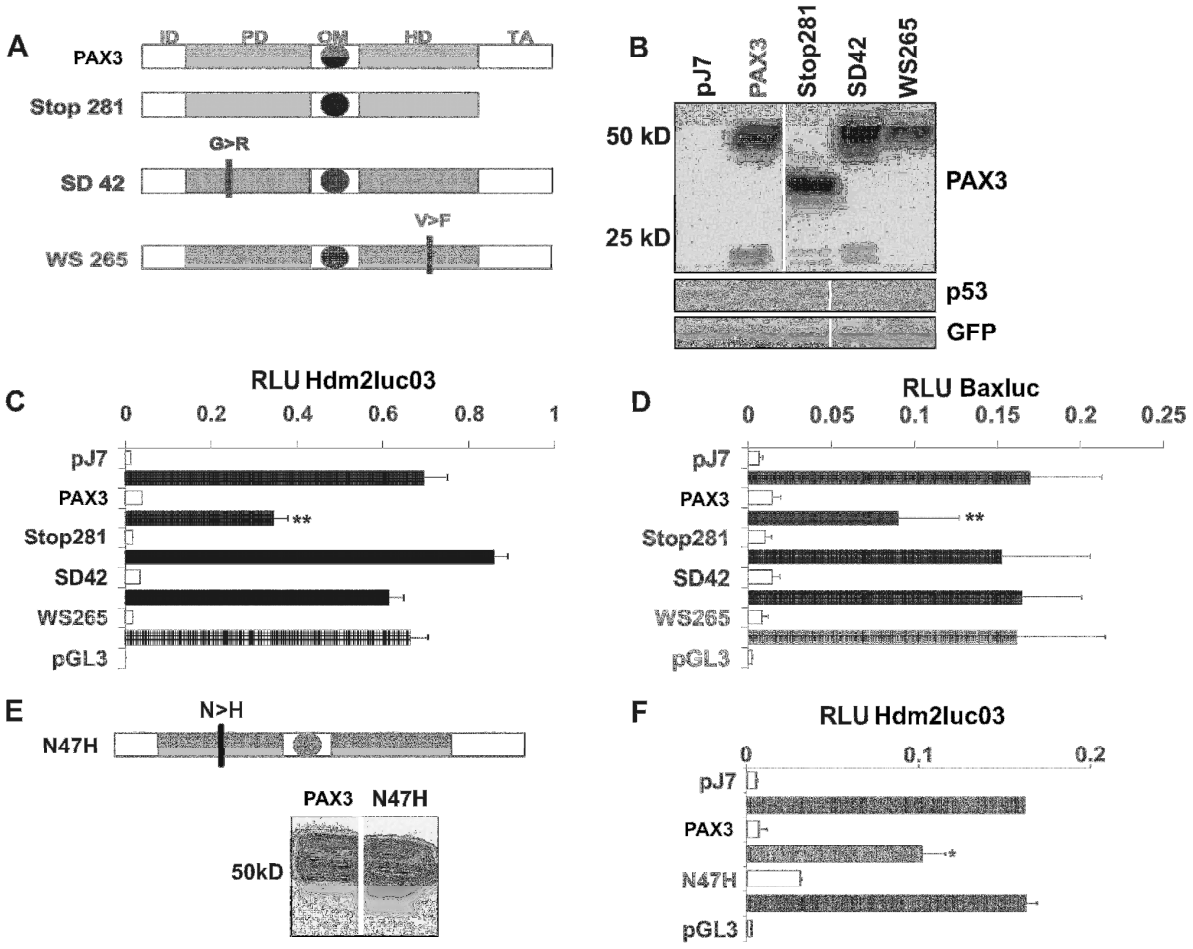
Fig. 3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