NEDDylation regulates E2F-1-dependent transcription

Sarah J. Loftus, Geng Liu, Simon M. Carr, Shonagh Munro & Nicholas B. La Thangue⁺

Laboratory of Cancer Biology, Department of Oncology, University of Oxford, Oxford, UK

The ubiquitin-like molecule NEDD8 modifies cullin-RING ubiquitin E3 ligases. NEDD8 has been shown to have a few additional substrates, but the extent to which this modification targets non-cullins and the functional significance of such modifications remain unclear. Here, we demonstrate that the cell-cycle-regulating transcription factor E2F-1 is a substrate for NEDD8 post-translational modification. NEDDylation results in decreased E2F-1 stability, lower transcriptional activity and slower cell growth. The lysine residues in E2F-1 targeted for NEDDylation can also be methylated, pointing to a possible interplay between these modifications. These results identify a new mode of E2F-1 regulation and highlight the emerging role of NEDD8 in regulating transcription factor stability and function.

Keywords: cell cycle; E2F-1; methylation; NEDD8

EMBO reports (2012) 13, 811–818. doi:10.1038/embor.2012.113

INTRODUCTION

It is now clear that the biological complexity of an organism is not simply a product of the number of genes it possesses. The 'histone code hypothesis', in which gene expression is mediated by the combination of post-translational modifications (PTMs) embellishing the surrounding histones, is now widely accepted. With the discovery that non-histone proteins are similarly subjected to PTM, it now seems possible that analogous 'protein codes' exist to mediate the activity of proteins such as transcription factors [1]. An example is the regulation of p53, which can be modified by methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and NEDDylation [2]. Interplay exists between these modifications so that some only exist in the absence or presence of others. The combination of modifications existing at any time can dictate which binding partners are recruited and thus whether activity is up- or downregulated.

Neural precursor cell-expressed developmentally down-regulated 8 (NEDD8), also known as Related to Ubiquitin 1 in yeast, is a member of the ubiquitin-like family of PTMs, which also includes the small ubiquitin-like modifier (SUMO). It is a 9 kDa protein that shares 80% sequence homology with ubiquitin [3].

NEDD8 is conjugated to its substrates in a three-step pathway analogous to that of ubiquitin, involving NEDD8 activation by an E1 (the APPBP-1-UBA3 heterodimer, also known as NEDD8-activating enzyme), transfer onto the catalytic cysteine of an E2 (either UBC12 or UBE2F) and then covalent attachment to the substrate with the aid of a substrate-specific E3 ligase [4]. The predominant and best-characterized NEDD8 substrates are the cullins, which are structural subunits of cullin-RING E3 ubiquitin ligases (CRLs). Cullin NEDDylation induces conformational changes within the CRL that enhance its binding to the E2 and decrease the distance between the E2 and the substrate recognition component, thereby increasing the proximity of ubiquitin (carried by the E2) to its target. Ubiquitin chain extension is also enhanced by cullin NEDDylation as it makes the nascent ubiquitin chain more accessible to the E2. Cullin NEDDylation further enhances CRL activity by hindering binding of the CRL inhibitor CAND1 [4]. More recently, a small number of non-cullin targets for NEDD8 have been identified [5–7]. Among this list of substrates are transcription factors such as p53 [8] and its family member Tap73β [9]. In both of these examples, NEDDylation impedes transcriptional activity.

The clinical relevance of NEDDylation has in recent years been demonstrated by the use of MLN4924, a small molecule inhibitor that targets NEDD8-activating enzyme by forming an adduct with NEDD8, which blocks the active site of the E1 [10]. Early laboratory studies showed the drug to have great potential as an antitumour agent, and clinical trials are at present underway to evaluate the role of MLN4924 in the treatment of haematological and solid tumour malignancies [11].

We reasoned that other cell-cycle regulators might be NEDDylated and focussed our attention on the E2F-1 transcription factor, which has a central role in mediating the cell cycle, in particular during the G1- to S-phase transition. It operates in a somewhat bipolar manner, exhibiting both oncogenic and tumour suppressor activities depending on the biological context [12,13]. Already E2F-1 is known to be methylated [14], phosphorylated [15], acetylated and ubiquitinated [16]. These properties of E2F-1 suggest that its activity might be subject to intricate mechanisms of control.

Here, we report that E2F-1 is regulated by NEDDylation. NEDDylation influences E2F-1 stability as well as transcriptional and growth control. Moreover, we provide evidence for interplay between NEDDylation and methylation. Our results thus hint towards a level of complexity that is consistent with an E2F-1 protein code.

Laboratory of Cancer Biology, Department of Oncology, University of Oxford, Old Road Campus Research Building, Old Road Campus off Roosevelt Drive, Oxford OX3 7DQ, UK

⁺Corresponding author. Tel: +44 1865 617090; Fax: 44 1865 617092;

E-mail: nick.lathangue@oncology.ox.ac.uk

Received 21 December 2011; revised 21 June 2012; accepted 2 July 2012; published online 27 July 2012

Fig 1 | E2F-1 is NEDDylated. (A) HA-E2F-1 (1 µg), p53 or empty vector were transfected with His-NEDD8 (4 µg) into H1299 cells and NEDD8 substrates isolated by His purification under denaturing conditions and subject to immunoblot analysis to show that E2F-1 is a substrate for NEDDylation. $n = 2$. (B) NEDD8 substrates were isolated from U2OS cells stably expressing His-NEDD8 using His purification under native conditions and analysed by immunoblotting. NTA-Ni²⁺ agarose beads in which the Ni²⁺ group was removed by EDTA were used as a control for nonspecific binding. $n = 2$. (C) HA-E2F-1 (1 µg) was transfected with His-NEDD8 (4 µg) into U2OS cells and cells treated with the indicated concentration of MLN4924 for 16 h. NEDD8 substrates were isolated by His purification under denaturing conditions and subject to immunoblot analysis. $n = 2$. (D) U2OS, SAOS2 and HeLa cells were treated for 16 h with MLN4924 (1 µM), lysed and subject to immunoblot analysis probing for endogenous E2F-1. $n = 4$. (E) NEDD8 substrates were isolated from H1299 cells co-expressing HA-E2F-1 (1 µg) and the NEDD8-specific protease NEDP1 (0.5, 1, 2 and 5 µg), using His purification under denaturing conditions and NEDD8-E2F-1 detected using immunoblot analysis (i). The decreasing level of NEDD8-E2F-1 with increasing NEDP1 was quantified and is shown graphically (ii). $n = 5$. (F) Immunoblot analysis of His-purified NEDD8 substrates from cells expressing the indicated E2F-1 mutants (1 µg) and His-NEDD8 (4 µg). The immunoblot (ii) is shown alongside a schematic (i) showing the mutants with respect to full-length E2F-1. $n = 2$. (G) Immunoblot analysis of His-purified NEDD8 substrates from cells expressing the indicated E2F-1 mutants (1 µg) and His-NEDD8 (4 µg). The immunoblot (ii) is shown together with a schematic (i) indicating the positions of the lysine residues, with red numbers representing lysine residues that were substituted for arginine. The level of NEDDylation of the mutants relative to wild-type is shown graphically. $n = 4$.

RESULTS

NEDDylation of E2F-1

We first expressed His-NEDD8 and E2F-1 in H1299 cells and used His purification followed by immunoblot analysis to identify E2F-1 as a substrate for NEDDylation, using a known NEDD8 substrate, p53, as a positive control [8] (Fig 1A). We then purified NEDD8 substrates from a stable His-NEDD8-inducible U2OS cell line under both native (Fig 1B) and denaturing (supplementary Fig S1A online) conditions and probed for endogenous E2F-1, which also indicated that E2F-1 is NEDDylated. When MLN4924 was used to treat U2OS cells ectopically expressing His-NEDD8 and E2F-1, E2F-1 NEDDylation was observed to decrease in a dose-dependent fashion (Fig 1C). This confirms that the observed NEDDylation on E2F-1 occurs via the classical NEDD8 pathway rather than an 'atypical' pathway in which components of the ubiquitin conjugation pathway can mediate NEDDylation [17,18]. Further support for E2F-1 NEDDylation via the typical conjugation pathway came from the observation that siRNA depletion of UBE1 had no effect on the level of E2F-1 NEDDylation (supplementary Fig S1Bi online). Moreover, a decrease in E2F-1 ubiquitination was observed in cells depleted of UBE1 (supplementary Fig S1Bii online). Furthermore, treating cells with MLN4924 caused an increase in endogenous E2F-1 levels in U2OS, SAOS2 and HeLa cells (Fig 1D), a result consistent with the NEDDylation of endogenous E2F-1.

NEDP1 (also known as DEN1 and SENP8) is a NEDD8-specific protease involved in processing the inactive NEDD8 precursor, exposing its diglycine motif so it might be conjugated onto its substrates, but it is also capable of removing NEDD8 from its substrates [19]. By co-expressing NEDP1 with E2F-1 and His-NEDD8, we observed a NEDP1 concentration-dependent decrease in the intensity of NEDD8-E2F-1 (Fig 1E). Further support for E2F-1 NEDDylation came from studying the effect of dominant-negative derivatives of the E2 ligases, UBE2F (C116S) [20] and UBC12 (C111S) [21] (supplementary Fig S1C online). A decrease in E2F-1 NEDDylation on expression of UBE2F (C116S) was observed, in a fashion dependent on the concentration of the E2 derivative.

NEDDylation occurs on lysine residues [22]. To identify residues targeted by NEDDylation, we generated a panel of mutant derivatives (truncations and substitution mutants) and assessed their ability to be NEDDylated (Fig 1F,G). We found

E2F-1 to be NEDDylated in its DNA-binding domain as the truncated mutants incorporating only residues 1–110 or 194–437 (namely mutants that did not include the DNA-binding domain) showed no evidence of NEDDylation (Fig 1F). Using substitution mutants in this region (residues 110–194), we identified lysine residues 182, 183, 185, 117, 120 and 125 to be required for efficient NEDDylation of E2F-1 (Fig 1G; supplementary Fig S1D online). The integrity of residue K185 appeared to be particularly important, as did residues K117, K120 and K125, as NEDDylation was decreased in mutants carrying substitutions in these residues. The K117/120R double mutant showed no decrease in NEDDylation with respect to wild-type E2F-1 (supplementary Fig S1D online) suggesting that either residue K125 is important for NEDDylation, or that, similar to the NEDD8 site on p53 [8], all three lysines need to be mutated to observe a decrease in NEDDylation. We therefore used K117/120/125R and K185R as NEDDylation-deficient mutants for further analysis.

NEDDylation affects transcriptional activity

We investigated whether NEDDylation impacted on the transcriptional activity of E2F-1 by co-expressing E2F-1 with luciferase reporter constructs and thereafter assessing the effect of NEDP1 and MLN4924 on promoter activity. An increase of promoter activity on NEDP1 expression or MLN4924 treatment was apparent (Fig 2A–D), suggesting that E2F-1 NEDDylation downregulates transcriptional activity. As the effect was recapitulated in the SAOS2 cell line, which has defective Rb and p53 (Fig 2D), we could rule out the possibility that the observed effects of MLN4924 treatment were mediated by NEDDylation of p53 or by the interaction of Rb with E2F-1, although we cannot, as yet, rule out any influence by other E2F-1-binding partners such as DP-1 and p300. The hypothesis that E2F-1 NEDDylation downregulates its transcriptional activity was further supported by the observation that the K185R and K117/120/125R mutant derivatives had higher levels of reporter activity than the wild-type (Fig 2E). This effect did not arise from any perturbations in intracellular localization resulting from the residue substitution, as both E2F-1 mutants had a nuclear location (supplementary Fig S1E online). Further, when His-NEDD8 was ectopically expressed, a reduction in p73 promoter activity was observed (Fig 2F). The increased transcriptional activity that occurred on the expression of NEDP1 reflected enhanced E2F-1 DNA-binding activity, detected by chromatin

immunoprecipitation (ChIP), and was not apparent with the K185R mutant, for which NEDP1 failed to augment DNA-binding activity (Fig 2G).

We used a His-NEDD8-inducible stable cell line to investigate whether NEDDylation influenced RNA and protein levels of E2F-responsive genes. We observed a decrease of dihydrofolate

reductase (DHFR), Cdc6 and p73 RNA, and E2F-1, Cdc6, Cdc2 and E2F-1 proteins on induction of His-NEDD8 expression (Fig 2H), providing further support that NEDDylation downregulates E2F-1 activity. These results were recapitulated by treating cells with MLN4924, which caused an increase in expression of E2F-1 target genes (Fig 2I), which might at least in part result from the

Fig 2 | E2F-1 NEDDylation influences its transcriptional activation. (A–F) E2F-1 (100 ng), NEDP1 (200 ng) and His-NEDD8 (500 ng) (where indicated) were co-expressed in U2OS (A–C; E,F) or SAOS2 (D) cells with the indicated luciferase reporter construct (Cdc6-luciferase or p73-luciferase; 100 ng) and β -gal (100 ng) and where indicated treated with MLN4924 (1 μ M) for 16 h. E2F-1 promoter activity was measured as described and input levels analysed by immunoblotting. For E, quantification and normalization of E2F-1 input level (normalized to actin) showed the ratio of WT:K185R:K117/120/125R to be 1:1.1:0.7. (** $P < 0.01$, * $P < 0.05$, Student's t -test) $n = 4$. (G) ChIP from U2OS cells expressing either wild-type E2F-1 or K185R (1 μ g) and NEDP1 (1 μ g) as indicated (i). An immunoblot of ectopic protein levels is also shown (ii). When quantified, NEDP1 input level fluctuates by 6% between wild-type and K185R treatments. (iii) Quantification of E2F-1 binding to Cdc6 and E2F-1 promoters by real-time PCR. (** $P < 0.01$, Student's t -test) $n = 4$. (H) RNA was purified from stable inducible His-NEDD8-expressing U2OS cells and RT-PCR performed to measure the level of the indicated RNA species (i). The decrease in DHFR and p73 levels are significant to a 1% confidence level and that of Cdc6 is significant to a 5% confidence level (Student's t -test). Protein levels of the indicated E2F target genes in these cells are shown in (ii). The decrease in Cdc2, DHFR and E2F-1 levels is significant to a 1% confidence level and that of Cdc6 significant to a 5% confidence level (Student's t -test). Protein inputs for NEDD8 are shown in (iii). $n = 4$. (I) RNA was purified from U2OS cells and RT-PCR performed to measure the level of the indicated RNA species following treatment with MLN4924 (1 μ M) for 16 h. The observed increases in DHFR and p73 RNA were significant to a 5% confidence level. $n = 3$. In all panels, error bars represent s.e.'s. ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcriptase PCR; WT, wild-type.

increased E2F-1 levels observed on MLN4924 treatment (Fig 1D). Thus, NEDDylation influences the transcriptional and DNA-binding properties of E2F-1.

NEDDylation regulates stability and growth control

NEDDylation has previously been shown to regulate the stability of its substrates [5,6]. To investigate this possibility for E2F-1, we compared the level of His-tag-purified NEDDylated E2F-1 with the level of total ectopic E2F-1 at several time points following cyclohexamide treatment and observed that E2F-1 is destabilized on NEDDylation (Fig 3A). This effect was also apparent with NEDP1, because E2F-1 in NEDP1-expressing cells had an extended half-life (Fig 3B). It is also consistent with this effect that the K185R and K117/120/125R mutants had an increased half-life (Fig 3B).

As E2F-1 is known to be stabilised under DNA damage [23], we investigated the effect of etoposide and doxorubicin treatment on E2F-1 NEDDylation and found NEDDylated E2F-1 levels to decrease by more than 80% under these conditions (Fig 3C). Further, treatment with MLN4924 no longer had the effect of increasing endogenous E2F-1 levels upon DNA damage (Fig 3D). These results suggest that E2F-1 NEDDylation is regulated during the DNA damage response.

As E2F-1 is important for growth control, we reasoned that NEDDylation could affect cell growth. We therefore expressed wild-type E2F-1 and its mutants, and thereafter assessed their activity in a cell proliferation assay (Fig 3E). Very interestingly, U2OS cells expressing the NEDDylation-deficient K185R and K117/120/125R mutants proliferated faster than those expressing wild-type E2F-1, suggesting that NEDDylation impacts on the growth-regulating properties of E2F-1.

Interplay with E2F-1 NEDDylation

Our results indicate that residue K185 is important for efficient NEDDylation of E2F-1. We found the identification of K185 to be provocative because the same residue is methylated by Set7/9 (Fig 4A–C) [14]. We surmised, therefore, that there might be a level of interplay between methylation and NEDDylation of E2F-1. To explore this idea, we established conditions for measuring Set7/9-dependent methylation of K185, which was apparent both *in vitro* and in cells (Fig 4A–C). Under conditions of K185 methylation in cells, we depleted Set7/9 and thereafter monitored

the level of E2F-1 NEDDylation. Strikingly, depletion of Set7/9 reduced the level of wild-type E2F-1 NEDDylation, with a lesser effect evident on the K185R mutant (Fig 4D). At the transcriptional level, in Set7/9 depleted cells E2F-1 failed to respond to NEDP1 (Fig 4E). Furthermore, K185R exhibited enhanced transcriptional activity and, when compared with wild-type E2F-1, was less responsive to NEDP1 (Fig 4F). These results suggest a regulatory role for methylation of E2F-1 that is mediated in part through an influence on site-specific NEDDylation.

DISCUSSION

We have demonstrated that E2F-1 is NEDDylated, that NEDDylation negatively regulates E2F-1 activity and that there exists a level of crosstalk between NEDDylation and methylation. Our results are somewhat analogous to those seen for p53, where NEDDylation requires a tetrapeptide motif consisting of three lysines and a serine residue (similar to the E2F-1 NEDDylation site), and results in attenuation of its transcriptional activity [8]. Residue K372 of p53 is both required for NEDDylation and is a target for Set7/9 [24], thus raising the interesting possibility that protein regulation by NEDDylation and methylation by Set7/9 are closely linked events. In addition, BCA3 NEDDylation has been shown to affect transcription, with BCA3 NEDDylation resulting in suppression of NF κ B transcription via its ability to bind the p65 and cyclin D1 promoter [25]. Perhaps, therefore, NEDDylation has a more general role in gene control, reflecting a combination of its direct regulation of transcription factors and enhanced ubiquitination from its role in CRL activity.

There are also other reported examples of NEDDylation influencing protein stability [5,6]. A noteworthy example is the observation of MDM2 stabilization by NEDDylation [26]. DNA damage was found to increase NEDP1 levels which reversed MDM2 stabilization, causing a rise in p53 level and apoptosis. This is in support of our observations that NEDP1 reduces E2F-1 NEDDylation and that E2F-1 NEDDylation decreases under conditions of DNA damage. In addition, as we have observed many parallels with E2F-1 and p53 NEDDylation, it is plausible that NEDDylation provides a link between the E2F-1 and p53 cell-cycle regulatory pathways. The interesting observation that the NEDDylation and Set7/9 methylation sites on both E2F-1 and p53 overlap raises the possibility that a wider interplay between

Fig 3 | E2F-1 NEDDylation results in its destabilisation. (A) U2OS cells transfected with expression vectors encoding HA-E2F-1 (1 μ g) and His-NEDD8 (4 μ g) were treated with cyclohexamide (100 μ M) and harvested at time points over 6 h. NEDD8-E2F-1 was isolated by His purification under denaturing conditions and the results analysed by immunoblotting, to compare the half-life of NEDDylated E2F-1 with total ectopic E2F-1. Quantification showed that NEDD8-E2F-1 had a half-life that was 32% shorter than that of total E2F-1. $n = 2$. (B) U2OS cells expressing ectopic NEDP1 (2 μ g) and HA-E2F-1, K185R or K117/120/125R (1 μ g) were harvested over 4 h following cyclohexamide treatment and analysed by immunoblotting. Quantification is shown alongside. $n = 2$. (C) NEDD8 substrates were purified from U2OS cells ectopically expressing His-NEDD8 and HA-E2F-1, and treated with either etoposide (20 μ M) or doxorubicin (2 μ M) for 16 h, and the level of NEDD8-E2F-1 analysed by immunoblotting. $n = 3$. (D) U2OS cells were treated with MLN4924 (1 μ M), etoposide (20 μ M) or doxorubicin (2 μ M) for 16 h and lysates subject to immunoblotting, probing for endogenous E2F-1. (E) U2OS cells were transfected with either wild-type, K185R or K117/120/125R E2F-1 (1 μ g), and cell colonies stained with crystal violet after 10 days (i). Quantification showed colony numbers to be as follows: pcDNA3 171; wild-type 120; K185R 148 and K117/120/125R 225. The rate of proliferation of transfected cells was measured by counting cells every 2 days for 10 days after staining with trypan blue and is presented graphically in (ii). s.e.'s are representative of three independent repeats. Protein levels at the end of the 10-day period are shown in (iii). $n = 3$. GFP, green fluorescent protein; WT, wild-type.

Fig 4 | E2F-1 methylation at K185 may be required for its NEDDylation. (A,B) Recombinant E2F-1 (1 µg) (A) or peptides (5 µg), either methylated (185me) or unmethylated (185) (B), were incubated with Set7/9 (0.5 µg) in the presence of ³H-labelled SAM. Substrate methylation was quantified by measuring the transfer of radiolabel, which was detected in a filter assay. *n* = 5. In all panels, error bars represent s.e.'s. (C) HA-E2F-1 (4 µg) was immunoprecipitated from U2OS cells with either anti-HA or control (–) antibody, and analysed by immunoblotting with either anti-E2F-1 or a site-specific methyl-K185 antibody. Input (In) levels are also shown. *n* = 2. (D) NEDD8 substrates were purified under denaturing conditions from H1299 cells treated with Set7/9 (S) or control (C) siRNA (20 nM) under ectopic HA-E2F-1 (1 µg) and His-NEDD8 (4 µg) expression. The results were analysed by immunoblotting. *n* = 3. (E) E2F-1 (100 ng) and NEDP1 (200 ng), as indicated, were co-expressed in U2OS cells with Cdc6-luciferase reporter construct (100 ng) and β-gal (100 ng) following treatment with either Set7/9 (S) or control (C) siRNA (20 nM). Promoter activity was measured as described. Immunoblot analysis of protein expression level is shown. (**P* < 0.05, Student's *t*-test) *n* = 5. (F) Either E2F-1 or K185R (100 ng) were co-expressed with NEDP1 (200 ng), as indicated, with Cdc6-luciferase reporter (100 ng) and β-gal (100 ng) plasmids and promoter activity measured as described. Immunoblot analysis of the protein expression level is also shown. (***P* < 0.01, Student's *t*-test) *n* = 5. In panels E and F, error bars represent s.e.'s. (G) Proposed model of the interplay between methylation and NEDDylation. It is suggested, based on the results presented, that Set7/9 methylation of E2F-1 influences subsequent NEDDylation of E2F-1, which then impacts on the biochemical and functional properties of E2F-1. IP, immunoprecipitation; SAM, S-adenosyl-L-methionine; siRNA, short interfering RNA; WT, wild-type.

transcription factor regulation by these modifications exists, and this should be investigated further.

METHODS

His purification under denaturing conditions. Cells were treated with MG132 (20 µM) (Calbiochem) 4 h before harvesting and lysates prepared and His-NEDD8 substrates purified

using Ni²⁺-NTA agarose beads (Qiagen) according to the manufacturer's instructions but with several modifications as outlined in the supplementary information online.

Luciferase reporter assays. H1299 cells were plated onto 6 cm dishes and transfected as indicated, harvested after 48 h and lysed in 100 µl Reporter Lysis Buffer (Promega). Fifty percent of lysate was used to measure luciferase, 10% to measure β-gal

(absorbance at 420 nm read after addition of buffer to a final concentration of 100 mM Na₂PO₄ (pH7.3), 1 mM MgCl₂, 50 mM β-mercaptoethanol and 0.665 mg/ml ONPG and incubation at 37°C) and 40% lysate analysed by immunoblot to measure protein levels. Luciferase values were normalized according to the β-gal reading.

Chromatin immunoprecipitation. Chromatin was prepared as described previously [27]. The recovered DNA was analysed by real-time PCR as described [28] on the MX3005P QPCR system (Stratagene) using Brilliant II SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. Primer sequences are available on request.

Reverse transcriptase PCR. RNA was isolated from cells with the use of Trizol (Invitrogen). Five microgram of total RNA was used to generate complementary DNA using an oligo(dT)₂₀ primer and Superscript III reverse-transcriptase enzyme (Invitrogen). Two microlitre of this reaction was subsequently used as a template for PCR using gene-specific primers (sequences available on request) and Paq 5000 DNA polymerase (Agilent Technologies).

In vitro methylation assay. *In vitro* methylation assays were carried out using bacterially expressed recombinant GST-E2F-1 or GST-K185R E2F-1 as described previously [29].

Further Methods can be found in supplementary information online.

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

ACKNOWLEDGEMENTS

We thank Sarah Atkinson for her help in preparation of the figures for the manuscript. This work was funded by Cancer Research UK, Medical Research Council and Leukaemia & Lymphoma Research. We thank Linacre College, Oxford, for the J.R.F. awarded to S.M.C.

Author contributions: S.J.L., G.L., S.M.C. and S.M. undertook all experimental work, S.J.L. and N.B.L.T. wrote the manuscript and N.B.L.T. directed the project.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Sims RJ III, Reinberg D (2008) Is there a code embedded in proteins that is based on post-translational modifications? *Nat Rev Mol Cell Biol* **9**: 815–820
2. Dai C, Gu W (2010) p53 post-translational modification: deregulated in tumorigenesis. *Trends Mol Med* **16**: 528–536
3. Herrmann J, Lerman LO, Lerman A (2007) Ubiquitin and ubiquitin-like proteins in protein regulation. *Circ Res* **100**: 1276–1291
4. Watson IR, Irwin MS, Ohh M (2011) NEDD8 pathways in cancer, Sine Quibus Non. *Cancer Cell* **19**: 168–176
5. Oved S et al (2006) Conjugation to Nedd8 instigates ubiquitylation and down-regulation of activated receptor tyrosine kinases. *J Biol Chem* **281**: 21640–21651
6. Sundqvist A, Liu G, Mirsalotis A, Xirodimas DP (2009) Regulation of nucleolar signalling to p53 through NEDDylation of L11. *EMBO Rep* **10**: 1132–1139
7. Ryu JH, Li SH, Park HS, Park JW, Lee B, Chun YS (2011) Hypoxia-inducible factor alpha subunit stabilization by NEDD8 conjugation is reactive oxygen species-dependent. *J Biol Chem* **286**: 6963–6970
8. Xirodimas DP, Saville MK, Bourdon JC, Hay RT, Lane DP (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* **118**: 83–97
9. Watson IR, Blanch A, Lin DC, Ohh M, Irwin MS (2006) Mdm2-mediated NEDD8 modification of TAp73 regulates its transactivation function. *J Biol Chem* **281**: 34096–34103
10. Soucy TA, Dick LR, Smith PG, Milhollen MA, Brownell JE (2010) The NEDD8 conjugation pathway and its relevance in cancer biology and therapy. *Genes Cancer* **1**: 708–716
11. Soucy TA, Smith PG, Rolfe M (2009) Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer. *Clin Cancer Res* **15**: 3912–3916
12. Sahin F, Sladek TL (2010) E2F-1 has dual roles depending on the cell cycle. *Int J Biol Sci* **6**: 116–128
13. Johnson DG, Degregori J (2006) Putting the oncogenic and tumor suppressive activities of E2F into context. *Curr Mol Med* **6**: 731–738
14. Kontaki H, Talianidis I (2010) Lysine methylation regulates E2F1-induced cell death. *Mol Cell* **39**: 152–160
15. Stevens C, Smith L, La Thangue NB (2003) Chk2 activates E2F-1 in response to DNA damage. *Nat Cell Biol* **5**: 401–409
16. Galbiati L, Mendoza-Maldonado R, Gutierrez MI, Giacca M (2005) Regulation of E2F-1 after DNA damage by p300-mediated acetylation and ubiquitination. *Cell Cycle* **4**: 930–939
17. Hjerpe R, Thomas Y, Chen J, Zemla A, Curran S, Shpiro N, Dick LR, Kurz T (2012) Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes. *Biochem J* **441**: 927–936
18. Leidecker O, Matic I, Mahata B, Pion E, Xirodimas DP (2012) The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions. *Cell Cycle* **11**: 1142–1150
19. Mendoza HM, Shen LN, Botting C, Lewis A, Chen J, Ink B, Hay RT (2003) NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. *J Biol Chem* **278**: 25637–25643
20. Huang DT et al (2009) E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification. *Mol Cell* **33**: 483–495
21. Wada H, Yeh ET, Kamitani T (2000) A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo. *J Biol Chem* **275**: 17008–17015
22. Vertebral AC (2011) Uncovering ubiquitin and ubiquitin-like signaling networks. *Chem Rev* **111**: 7923–7940
23. Blattner C, Sparks A, Lane D (1999) Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53. *Mol Cell Biol* **19**: 3704–3713
24. Chuikov S et al (2004) Regulation of p53 activity through lysine methylation. *Nature* **432**: 353–360
25. Gao F, Cheng J, Shi T, Yeh ET (2006) Neddylation of a breast cancer-associated protein recruits a class III histone deacetylase that represses NFκB-dependent transcription. *Nat Cell Biol* **8**: 1171–1177
26. Watson IR, Li BK, Roche O, Blanch A, Ohh M, Irwin MS (2010) Chemotherapy induces NEDP1-mediated destabilization of MDM2. *Oncogene* **29**: 297–304
27. Zalmas LP, Zhao X, Graham AL, Fisher R, Reilly C, Coutts AS, La Thangue NB (2008) DNA-damage response control of E2F7 and E2F8. *EMBO Rep* **9**: 252–259
28. Jansson M, Durrant ST, Cho EC, Sheahan S, Edelmann M, Kessler B, La Thangue NB (2008) Arginine methylation regulates the p53 response. *Nat Cell Biol* **10**: 1431–1439
29. Carr SM, Munro S, Kessler B, Oppermann U, La Thangue NB (2011) Interplay between lysine methylation and Cdk phosphorylation in growth control by the retinoblastoma protein. *EMBO J* **30**: 317–327