Non-degradative ubiquitination of the Notch1 receptor by the E3 ligase MDM2 activates the Notch signalling pathway

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The Notch receptor is necessary for modulating cell fate decisions throughout development, and aberrant activation of Notch signalling has been associated with many diseases, including tumorigenesis. The E3 ligase MDM2 (murine double minute 2) plays a role in regulating the Notch signalling pathway through its interaction with NUMB. In the present study we report that MDM2 can also exert its oncogenic effects on the Notch signalling pathway by directly interacting with the Notch 1 receptor through dual-site binding. This involves both the N-terminal and acidic domains of MDM2 and the RAM [RBP-Jκ (recombination signal-binding protein 1 for Jκ)-associated molecule] and ANK (ankyrin) domains of Notch 1. Although the interaction between Notch1 and MDM2 results in ubiquitination of Notch1, this does not result in degradation of Notch1, but instead leads to activation of the intracellular domain of Notch1. Furthermore, MDM2 can synergize with Notch1 to inhibit apoptosis and promote proliferation. This highlights yet another target for MDM2-mediated ubiquitination that results in activation of the protein rather than degradation and makes MDM2 an attractive target for drug discovery for both the p53 and Notch signalling pathways.

Key words: murine double minute 2 (MDM2), non-degradative ubiquitination, Notch1, NUMB, transcriptional activation.

INTRODUCTION

During normal development and homeostasis the Notch receptor is an important regulator of cellular differentiation, proliferation and apoptotic events, and directs the cell fate selection of cells [1,2]. There are four mammalian Notch receptors (Notch 1–4), which have broad expression patterns throughout mammalian tissues. Notch is a membrane protein that undergoes at least three critical proteolytic steps that are required for its maturation. Initially, a furin-like convertase cleaves Notch into two distinct fragments that interact to form a functional heterodimer on the cell surface. Notch then binds to the transmembrane ligands of the Delta-like and Jagged ligands on neighbouring cells. Upon ligand binding, a second cleavage event occurs by the ADAMs (a disintegrin and metalloproteinases), leading to the release of the Notch extracellular domain. A γ-secretase, whose activity is dependent on the presenilins, carries out the final cleavage to release the active NICD (Notch intracellular domain). NICD then translocates to the nucleus where it acts as a co-transactivator with the transcription factors RBP-Jκ (recombination signal-binding protein 1 for Jκ) [CSL (CBF1/suppressor of Hairless/Lag-1)/CBF1] and Mastermind to generate a large transcriptional activator complex to modulate transcription of downstream target genes, such as the Hes/Hey family of transcription factors [1–3].

Genetic abnormalities in components of the Notch signalling pathway have been implicated in a number of diseases, including neurodegenerative and developmental disorders and many cancers [4]. Notch1 and Notch4 have been shown to contribute to the development of mammary carcinoma and overexpression of the active forms of Notch1 and Notch4 has been shown to be involved in lung and cervical cancers, melanoma, neuroblastoma and T-ALL (T-cell acute lymphoblastic leukaemia) [5]. Genetic evidence suggests that Notch1 can co-operate with oncogenes in the cell to promote tumorigenesis. In all cases where Notch is associated with tumorigenesis, it appears that the intracellular domain is involved.

Post-translational modifications, such as glycosylation, phosphorylation and ubiquitination, affect activation of the Notch pathway [2]. During the ubiquitination process, ubiquitin is attached to an ubiquitin-activating enzyme (E1) and subsequently transferred to an ubiquitin-conjugating enzyme (E2). The ubiquitin ligase (E3) transfers the ubiquitin to a specific protein substrate [6]. Ubiquitin targets proteins for degradation by the proteosome, but can also serve as a signal for receptor internalization and trafficking to multivesicular bodies and the lysosome [6]. Recent evidence suggests a function for ubiquitination in transcriptional regulation and modification [7].

NUMB protein is a major regulator of the Notch pathway [8]. NUMB recruits components of the ubiquitination machinery to the Notch receptor, resulting in Notch1 ubiquitination and degradation of the intracellular domain. This prevents nuclear translocation of Notch and downstream activation of target genes [8]. NUMB promotes the ubiquitination and degradation of Notch1 through an interaction with the cytosolic HECT (homologous to E6-AP C-terminus) domain-containing E3 ligase Itch [9]. The PTB (phosphotyrosine-binding) domain of NUMB is required for both Notch1 ubiquitination and down-regulation of Notch1 nuclear activity [9]. Aberrant activation of Notch has been demonstrated in over 50% of breast cancers, as a result of the ubiquitination and degradation of NUMB [10,11].

In addition, several other E3 ubiquitin ligases have been shown to regulate the Notch receptor. In Drosophila, genetic
Evidence suggests that another HECT-domain-containing E3 ubiquitin ligase, Suppressor of deltex, negatively regulates Notch receptor signalling [12]. Phosphorylation of Notch in the PEST domain leads to binding of the F-box protein, Fbw7, part of a SCF (Skp1/cullin/F-box) ubiquitin ligase complex, resulting in proteasomal degradation of Notch [13].

The oncogene and E3-ubiquitin ligase MDM2 (murine double minute 2) is a well-known regulator of many components of the p53 tumour-suppressor pathway, including Numb [14–16]. In a similar manner to p53, MDM2 targets Numb for ubiquitination and degradation using a dual-site mechanism, involving MDM2 N-terminal and AD (acidic domain) interactions with the PTB domain of Numb [17]. In the present study, we demonstrate that MDM2 also regulates the Notch signalling pathway by a direct interaction with NICD1 that results in ubiquitination. However, ubiquitination of NICD1 does not appear to target it for proteasomal degradation, but instead leads to activation of the Notch signalling pathway. Therefore MDM2 can exert its oncogenic effects on the Notch signalling pathway in two ways: (i) by interacting with and degrading the Notch regulator Numb; and (ii) by forming a direct complex with Notch1 and activating its downstream targets. This makes MDM2 an attractive target for drug discovery for both the p53 and Notch pathways, especially in cancers where aberrant activation of Notch commonly occurs.

**EXPERIMENTAL**

**Antibodies, plasmids and peptides**

pcDNA3.1-MDM2, pcMV-His-ubiquitin and pT7.7/MDM2 were gifts from Professor David Lane (p53 Lab, A*STAR, Immunos, Singapore). pcDNA3.1-NUMB and pGEX2T-NUMB were gifts from Professor Moshe Oren (Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel). GST (glutathione transferase)-MDM2, GST–ΔN MDM2, GST–ΔAD MDM2, GST–AD MDM2 and GST–N MDM2 have been described previously [18]. His–MDM2-RING has also been described previously [23]. pFN2A-NUMB, NUMB PTB and NUMB ΔPTB have been described previously [17]. The Δ2x RBP-Jκ reporter plasmid was a gift from Dr Sally Lowell (MRC Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, U.K.). GFP (green fluorescent protein)–NICD1 was purchased from Addgene, which contained a mutation. The mutation was corrected using site-directed mutagenesis (Stratagene) and the plasmid was sequenced for confirmation. This plasmid was then used to subclone into the pcDNA3.1, pENTR11 (Gateway, Invitrogen) and pTRCHis plasmids using the following primers: pcDNA3.1 forward, 5′-CTAGGATCCTAGCCGCCACCCATGGGCAACACGCCCTGCT-3′; pTRCHis/pENTR11 forward, 5′-CTAGGATCCTAGCCGCCACCCATGGGCAACACGCCCTGCT-3′ and reverse 5′-CTAGGATCCTAGCCGCCACCCATGGGCAACACGCCCTGCT-3′; Goat anti-NUMB (Abcam; Ab 4147), anti-Notch1 (Millipore; MAB5352) and rabbit anti-Hes1 (Santa Cruz Biotechnology; H-140) antibodies were used to detect NUMB, NICD1 and Hes1 respectively. Monoclonal antibodies 4B2 and 2A10 were used to detect MDM2, and DO-1 antibody was used to detect p53 (gifts from Professor T. Hupp, Edinburgh Cancer Research Centre, University of Edinburgh, Edinburgh, U.K.). Anti-GST and anti-β-actin antibodies were from Sigma. Biotin-labelled peptides were synthesized by Mimotopes. HIV Tat fluorescent-labelled peptides were from Mimotopes. (NUMB peptide 9: YICRDGTTRWICHCFMAV-YGRKKRRQRRR and BOX-V: SYLLPGMASF-YGRKKRRQRRR).

**Cell culture and immunobLOTS**

H1299, MDA-MB-468, T47D, SKBR-3 and BT-474 cells were maintained in RPMI 1640 medium (Invitrogen) MCF7, ZR75 and A375 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) (Invitrogen). Medium was supplemented with 10% FBS (fetal bovine serum) (Biosera) and 1% penicillin/streptomycin (Invitrogen) and cells were grown at 37°C and 5% CO₂. The MCF7 stable cell line was generated using pcDNA3.1-MDM2 plasmid and pcDNA3.1 empty vector (Invitrogen) through geneticin-resistance selection. The cells were transfected at 80% confluency using Lipofectamine™ 2000 (Invitrogen) or Attractene (Qiagen) following the manufacturer’s instructions and DNA was equalized by the addition of empty vector control. siRNA (small interfering RNA) (Dharmacon) was transfected into cells using Attractene following the manufacturer’s instructions. Peptides were added to the cells as indicated in the Figure legends and incubated overnight. Cells were lysed in a buffer system composed of 50 mM HEPES, pH 7.6, 1% Nonidet P40, 150 mM KCl, 1 mM EDTA, 0.5 mM DTt (dithiothreitol) and protease inhibitors (Roche) and immunoblots were carried out as described previously [19].

**Immunoprecipitation**

MCF7 cells were harvested and lysed on ice in 50 mM HEPES, pH 7.6, 1% Nonidet P40, 150 mM KCl, 1 mM EDTA, 0.5 mM DTt and protease inhibitor cocktail (Roche). Extracts were clarified by centrifugation steps. A total of 2 μg of 4B2 (anti-MDM2) antibody was added to the clarified extracts, as well as to a buffer control, and incubated for 1 h with rotation, at 4°C. Pre-washed Protein G–Sepharose (GE Healthcare) was added to the samples and incubated for an additional 1 h. The resin was washed twice with lysis buffer and three times with PBS. Samples were eluted by boiling in SDS sample buffer and analysed by immunoblotting with anti-MDM2 (2A10), anti-NUMB and anti-NICD1 antibodies.

**Protein purification**

MDM2 was expressed and purified from *Escherichia coli* as described previously [20]. GST–MDM2, GST–NICD1 and GST–NUMB constructs were transferred into *E. coli* BL21 (DE3) cells and purified on glutathione–Sepharose beads (GE Healthcare) following the manufacturer’s instructions. The GST tag was cleaved using PreScission Protease (GE Healthcare) following the manufacturer’s instructions. His₈–NICD1 and His₈–MDM2-RING were transferred from the pTRCHisC-NICD1 and pTRCHis-MDM2 RING plasmids respectively into *E. coli* BL21 (DE3) cells. Colonies were picked and grown overnight in LB (Luria–Bertani)/ampicillin at 37°C. Fresh culture medium was inoculated and the culture was grown to D = 0.6 and induced with IPTG (isopropyl β-D-thiogalactopyranoside) (1 mM) for 3 h at 37°C. The pellet was resuspended in lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P40, 10% (v/v) glycerol, 10 mM MgCl₂, and 20 mM imidazole) containing lysozyme and protease inhibitor cocktail (Roche) and incubated for 30 min on ice. The cell suspension was sonicated, centrifuged (10 min at 16000 g) and the supernatant was added to Ni-NTA (Ni²⁺-nitrilotriacetaete) beads (Qiagen) for 1 h at 4°C. Beads were washed and transferred to a 2.5 ml column (Mo Bi Tec). The protein was eluted with an imidazole gradient (0–250 mM) in buffer containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P40, 10% (v/v) glycerol and 10 mM MgCl₂. Fractions were analysed by
on ice for 30 min prior to adding to the plate. Readings were incubated with MDM2 or MDM2 was pre-incubated with NICD1 containing 0.1 μM sodium borate. Non-reactive sites were blocked using PBS containing 3% BSA. The wells were incubated with full-length MDM2 or NICD1 (as indicated in Figure legend) in PBS containing 0.25 mM benzamidine, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, ubiquitin (2 μg), E1 (UBE-1) (100 nM), E2 (UbcH5) (1 μM) and His6–NICD1 (250 ng). Reactions were started using purified MDM2 (50–100 ng), incubated for 30 min at 30°C and analysed using 4–12% NuPAGE gels in a Mops buffer system (Invitrogen) followed by immunoblot with anti-NICD1 antibody. Where peptides were used, they were added to the reaction mix prior to the addition of MDM2, as indicated in the Figure legend.

**ELISA assays**

For peptide ELISA, biotin-labelled peptides were captured on streptavidin-coated plates (25 μg/ml, Sigma) as detailed in the Figure legends. Non-reactive sites were blocked using PBS containing 3% BSA. The wells were incubated with full-length MDM2 or MDM2 deletion mutants as indicated in the Figure legends, in PBS-0.1% BSA for 1 h at room temperature (22°C). The plates were washed with PBS containing 0.2% Tween and MDM2 binding was detected using the anti-MDM2 antibodies 2A10 or 4B2 and enhanced chemiluminescence. The protein ELISA assays were performed by coating white 96 well microtitre plates with the protein (as detailed in the Figure legends) in 0.1 M sodium borate. Non-reactive sites were blocked using PBS containing 3% BSA and the wells were incubated with MDM2 or NICD1 (as indicated in Figure legend) in PBS containing 0.1% BSA for 1 h at room temperature. Binding was detected using the appropriate primary antibodies and enhanced chemiluminescence. Competition ELISA assays were carried out as above, but NUMB wild-type or mutant proteins were pre-incubated with MDM2 or MDM2 was pre-incubated with NICD1 on ice for 30 min prior to adding to the plate. Readings were performed using Fluoroscan Ascent FL (Labsystems).

**Luciferase assay**

MCF7 and H1299 cells were plated into 6-well plates and transfected the following day using Attractene following the manufacturer’s instructions, with 12 μg RPB-Jx Reporter (10–100 ng) and/or Renilla luciferase (10–100 ng) and pcDNA3.1-NICD1(400 ng), pcDNA3.1-NUMB (400 ng) and pcDNA3.1-MDM2 (50 ng) as indicated in the Figure legends. Fluorescent peptides (10 and 20 μM) were added to the cells 7 h post-transfection (as indicated in the Figure legends) and incubated overnight. The Dual Luciferase Reporter Assay (Promega) was then performed on the cells following the manufacturer’s instructions. Readings were performed using Victor3 1420 Multilabel Counter (PerkinElmer).

**Caspase Glo 3/7 assay and cell proliferation assay**

Assays were performed using ZR75, A375 or H1299 cells as indicated in the Figure legends at approximately 80% confluency, in a 96-well format. Cells were transfected as indicated in the Figure legends using Attractene (Qiagen), following the manufacturer’s instructions. Cells were then left untreated or treated with Melphalan for 5 h (25 μM) or Cisplatin for 24 h (6 μM). Fluorescent peptides (10 and 20 μM) were added to the cells 7 h post-transfection and incubated overnight. Cells were processed the following day according to the Caspase-Glo 3/7 or CellTiter 96 Aqueous One Solution kit following the manufacturer’s protocol (Promega). Readings were performed using Victor3 1420 Multilabel Counter (Perkin Elmer).

**RESULTS**

**NICD expression is enhanced by MDM2 in cancer cells**

NUMB, a well-characterized regulator of Notch, was identified as an MDM2-binding partner using a yeast 2-hybrid screen and was later found to be a target for MDM2-catalysed ubiquitination and proteosomal degradation [15,16]. We have demonstrated previously an inverse correlation between the protein expression levels of NUMB and MDM2 in a panel of breast cancer cells and found that MDM2 could also degrade NUMB in breast cancer cells [17]. These results imply that MDM2 may contribute to the ubiquitination and degradation of NUMB observed in over 50% of breast cancer patients, which leads to aberrant activation of Notch signalling. To determine whether NICD1 was up-regulated in breast cancer cells expressing high levels of MDM2, we analysed the panel of breast cancer cells by Western blotting with an antibody that detects NICD1 (Figure 1A). Higher levels of activated Notch1 correlated with higher levels of MDM2 protein expression (Figure 1A). For example, MCF7 and ZR75 cells had high expression of NICD1 and MDM2, which correlated with the low NUMB levels we observed previously in these cells [17]. H1299 lung cancer cells were used as a control as we have found that they express high levels of NUMB and low levels of NICD1.

The effects of knockdown of MDM2 on the NUMB/Notch axis was then explored using siRNA. MCF7 cells were treated with siRNA against MDM2 and a decrease in MDM2 expression was observed (Figure 1B). Knockdown of MDM2 resulted in an increase in NUMB and p53 protein expression compared with the control siRNA (Figure 1B). Corresponding to these results, levels of activated Notch 1 (NICD1) decreased (Figure 1B).

Next, the half-life of NICD1 in MCF7 breast cancer cells with stably overexpressed MDM2 was measured. When these cells were treated with cyclohexamide, NICD1 levels were more stable in the presence of MDM2 (Figure 1C). In addition, a reduction in the half-life of NUMB was observed in cells with overexpressed MDM2 compared with control cells (Figure 1C). Interestingly, the reduction in NUMB protein levels was not observed until the 12 h time point, whereas NICD1 was already stabilized much earlier by 6 h, suggesting MDM2 regulates NICD1 and NUMB in a separate manner.

Considering the above results, MDM2 was then overexpressed in MCF7 or ZR75 breast cancer cells by transient transfection. An increase in protein expression of NICD1 was observed in the cells overexpressing MDM2 (Figure 1D). To overcome...
MDM2-mediated degradation of Numb and to restore Numb levels in cells, we transfected MDM2 into MCF7 and ZR75 cells in the presence of overexpressed Numb. As MDM2 elevated the levels of NIDC1 in both cell lines, even when Numb levels were high (Figure 1D), this suggests that MDM2 has some effect on NIDC1 which is independent from Numb. Hes1 is a downstream target of Notch and is an indicator that the Notch signalling pathway is activated. Indeed, Hes1 was up-regulated after transfection of the cells with MDM2, which was independent of Numb levels (Figure 1D). To determine whether the effect of MDM2 on NIDC1 levels was dependent on p53, H1299 cells (which are p53-null) were examined. A similar enhancement in NIDC1 levels was observed in these cells, even in the presence of overexpressed Numb (Figure 1E).

Nutlin is known to enhance levels of MDM2 in cells. Therefore, to increase the endogenous levels of MDM2, we treated ZR75 and MCF7 cells at different time points with Nutlin. Correlating with the enhancement of MDM2, an increase in NIDC1 levels was observed compared with control cells treated with DMSO (Figure 1F).

**MDM2 directly interacts with the intracellular domain of Notch**

As MDM2 appears to enhance levels of NIDC1 even when Numb degradation is not observed, we wanted to explore the possibility that MDM2 and NIDC1 could form a direct interaction. Initially, immunoprecipitation experiments were carried out to examine the MDM2–NIDC1 complex in cells. We have observed that MCF7 cells express low levels of Numb and higher levels of NIDC1 ([17] and Figure 1A). When MCF7 cells were immunoprecipitated with an antibody against MDM2 (4B2), we observed that MDM2 and NIDC1 were pulled down in a complex from cells (Figure 2A). However, Numb levels are low in this cell line and, indeed, we could not detect any Numb in the complex (Figure 2A), suggesting that MDM2 and NIDC1 form a direct interaction.

**Figure 1** MDM2 enhances the levels of NIDC1 in cells

(A) Panel of breast cancer cells (MCF7, MDA MB 468, ZR75, SKBR, T47D and BT-474) and a lung cancer cell line, H1299, immunoblotted for protein expression levels of NIDC1, MDM2 and β-actin loading control as indicated. (B) MCF7 cells treated with siRNA against MDM2 (200 nM) or control siRNA (ctr) (200 nM) as indicated, followed by immunoblotting with antibodies against Numb, p53, NIDC1, MDM2 and β-actin. Densitometry of the immunoblots is shown in the right-hand panel. (C) MCF7 cells stably overexpressing MDM2 or empty vector control were treated with cyclohexamide (50 μg/ml) for the times indicated (0–14 h), followed by immunoblotting for Numb, NIDC1, MDM2 and β-actin. (D) and (E) MCF7 and H1299 cells were transiently transfected with MDM2 (1 μg) in the presence or absence of Numb (1 μg). DNA was equalized by addition of empty vector control. Cells were analysed by immunoblotting with antibodies against Numb, NIDC1, Hes1, MDM2 and β-actin as indicated. The densitometry of the immunoblots is shown in the right-hand panel. (F) MCF7 and ZR75 cells were treated with 10 μM Nutlin (N) or DMSO control (D) for 8 or 12 h as indicated followed by immunoblotting with antibodies against MDM2, NIDC1 and β-actin. The densitometry of the immunoblots is shown below. Experiments presented in this Figure are representative of at least three separate experiments.
MDM2-mediated ubiquitination of NICD1

Figure 2  MDM2 forms a direct interaction with NICD1

(A) MCF7 cells were untransfected (UT) or transfected with NICD1 (1 μg) followed by immunoprecipitation with anti-MDM2 antibody (4B2) and analysed by immunoblotting with anti-MDM2 (2A10), anti-NICD1 and anti-NUMB as indicated. ctl, beads-only control; IP, immunoprecipitated cells; WCE, whole cell extracts. (B) Purified full-length GST–NICD1 (100 ng) or GST (100 ng) was immobilized on a 96-well microtitre plate and incubated with increasing amounts of purified full-length MDM2. Binding was detected with anti-MDM2 (2A10) antibody followed by chemiluminescence with an enhanced chemiluminescence reagent. (C) Schematic diagram depicting full-length, mutant and mini-domain MDM2 and NUMB proteins. ZF, zinc finger domain of MDM2. MDM2-ΔN is a mutant MDM2 missing the N-terminal hydrophobic pocket. NUMB-ΔPTB is missing the PTB domain of NUMB. NES, nuclear export signal; NLS, nuclear localization signal. (D and E) NICD1 or GST-only control protein (400 ng) were bound to a 96-well microtitre plate and incubated with 0–800 ng of purified N-terminus of MDM2 (MDM2-N) (D) or 0–4 μg FL MDM2 or MDM2 missing the N-terminus (MDM2-ΔN) (E). Binding was detected with anti-MDM2 (2A10) antibody followed by chemiluminescence with an enhanced chemiluminescence reagent. (F) NICD1 (400 ng) immobilized on a 96-well microtitre plate and incubated with increasing amounts of the AD of MDM2 (GST AD), MDM2 missing the AD (GST ΔAD) or GST control protein (GST) (0–4 μg) as indicated. Binding was detected with an anti-GST antibody followed by chemiluminescence with an enhanced chemiluminescence reagent. NICD1 or BSA (0–64 ng) were immobilized on a microtitre plate and incubated with the MDM2-RING domain (500 ng). Binding was detected with an anti-MDM2 antibody followed by chemiluminescence with an enhanced chemiluminescence reagent. MDM2-RING was also bound to the plate and incubated with anti-MDM2 antibody as a positive control. Experiments presented in this Figure are representative of at least three separate experiments. RLU, relative luciferase units.

Plate was coated with NICD1 protein, control GST protein or buffer only and incubated in the presence of increasing amounts of MDM2. MDM2 binding was analysed using an antibody against MDM2 (Figure 2B). MDM2 was found to form an interaction with NICD1 in vitro (Figure 2B). Next, we sought to determine the regions in MDM2 important for mediating the interaction with NICD1 using mini domain and mutant MDM2 proteins (Figure 2C). In a similar manner to other proteins such as p53 and NUMB, NICD1 was found to interact with the N-terminus of MDM2 (MDM2-N) in a protein–protein interaction.
assay, but not to the control GST-only protein (Figure 2D). To confirm the significance of the N-terminus of MDM2 as a binding interface for NICD1, a mutant form of MDM2 missing the N-terminus (MDM2-ΔN) was examined in this assay and a reduction in binding to NICD1 was observed compared with full-length MDM2 (Figure 2E). However, some degree of binding was still observed with this mutant protein missing the N-terminus, suggesting an additional binding site. As the AD of MDM2 is also a well-known binding site for a number of proteins, we tested the isolated AD (MDM2-AD) for NICD1 binding. The AD bound strongly to NICD1 (Figure 2F), but a reduction in binding was observed with the MDM2 mutant missing the AD (MDM2-ΔAD) (Figure 2F). To demonstrate that NICD1 binding was specific, we then tested NICD1 binding to the isolated RING domain of MDM2 in a protein–protein interaction assay. Indeed, no significant binding between NICD1 and the isolated RING domain of MDM2 was observed (Figure 2G), although the isolated RING domain when immobilized on the plate could bind to the MDM2 antibody (Figure 2G). These results imply that, in a similar manner to p53 and NUMB, MDM2 binds to NICD1 using a dual-site mechanism, involving specific N-terminal and AD interactions.

**Regions in NICD1 important for the interaction with MDM2**

The intracellular domain of Notch is composed of the RAM (RBP-Jk-associated molecule) domain, followed by an unstructured linker containing a nuclear localization signal and seven ANK (ankyrin) repeats. The C-terminus contains PEST motifs, which have been shown to be involved in providing degradation signals (Figure 3A). To determine which regions within NICD1 were important for MDM2 binding, biotin-tagged peptides of NICD1 were synthesized and bound to a streptavidin-coated microtitre plate. The peptides were incubated with full-length MDM2, purified from *E. coli* and binding was analysed by an anti-MDM2 antibody (Figure 3A). MDM2 bound to peptide 1 of NICD1, which encompasses the RAM domain of NICD1 (Figure 3A). In addition, a weaker interaction was observed with peptides 14 and 19 in the ANK domain (Figure 3A), although binding to these peptides was not always consistent, suggesting a weaker affinity for the ANK domain.

To analyse these results further, the peptides were examined for binding to the AD and N-terminus of MDM2. The AD seemed to bind strongly and consistently to the RAM domain peptide 1 (Figure 3B) and to another novel peptide, 26. However, the N-terminus of MDM2 did not interact with any of the peptides (Figure 3C), although binding to the p53 BOX-I control peptide was observed, suggesting the interaction with the N-terminus of MDM2 is a weaker interaction or perhaps requires a larger region of NICD1 to form a complex. These results suggest that MDM2 binds to NICD1 in an analogous manner to other proteins such as the transcription factor CSL, with the AD binding to the RAM domain of NICD1 and a weaker interaction formed with the ANK domain, perhaps also involving the N-terminus of MDM2.

**MDM2 disrupts the interaction between NICD1 and the PTB domain of NUMB**

The PTB domain of NUMB has been shown to be important for NUMB-mediated regulation of Notch and is reported to be involved in binding to NICD [9]. In addition, we have previously demonstrated that MDM2 binds to the PTB domain of NUMB [17]. To confirm the interaction of the NUMB PTB domain with NICD1, we carried out a protein–protein interaction assay using purified proteins (Figure 2C). NICD1 was found to bind to full-length NUMB and to the PTB domain of NUMB (PTB), but not to a mutant form of NUMB missing the PTB domain (ΔPTB) or to the GST-only control protein (Figure 4A). We have previously demonstrated that MDM2 binds to two peptides (8 and 9) within the NUMB PTB domain [17], suggesting an overlap in binding sites between NICD1 and MDM2. Therefore biotin-tagged peptides to the PTB domain of NUMB (peptides 7–9) were immobilized on a streptavidin-coated microtitre plate and examined for NICD1 binding (Figure 4B). NICD1 bound to peptides 8 and 9 within the PTB domain of NUMB (amino acids 113–148) (Figure 4B). NUMB peptide 14 is outwith the PTB domain and was used as a negative control in this experiment (Figure 4B). These results suggest that NICD1 and MDM2 bind to the same region on NUMB. In addition both the N-terminus and ADs of MDM2 are involved in the interactions with both NICD1 and NUMB. Therefore all three proteins may compete for binding to each other.

To investigate this further, NICD1 binding to MDM2 in the presence of NUMB or the PTB domain of NUMB was examined (Figure 4C). Both the full-length NUMB protein and the PTB domain of NUMB could compete with MDM2 for binding to NICD1 (Figure 4C). As both MDM2 and NICD1 bind to the NUMB PTB-domain-derived peptides 8 and 9, we next examined NICD1 binding to NUMB peptides in the presence of full-length MDM2, the AD of MDM2 or the GST-only protein control (Figures 4D and 4E). In the presence of full-length MDM2 the interaction between NICD1 and NUMB PTB domain peptides was reduced (Figures 4D and 4E). In fact, the isolated AD of MDM2 was sufficient to disrupt the interaction between NICD1 and NUMB peptides (Figures 4D and 4E). These results suggest that MDM2 can disrupt the NUMB–Notch complex and activate the Notch signalling pathway through yet another mechanism.

**NICD1 is ubiquitinated by MDM2**

As MDM2 forms a direct interaction with NICD1, we sought to determine whether NICD1 was a substrate for MDM2-mediated ubiquitination. A well characterized *in vivo* ubiquitination assay was employed, which has been extensively used to study p53 ubiquitination [21]. NICD1 was transfected into cells in the presence or absence of MDM2 and His₁₅₉-tagged ubiquitin, and a pull-down assay on nickel beads followed by immunoblot analysis was carried out. NICD1 was efficiently ubiquitinated in the presence of MDM2 (Figure 5A). Ubiquitination was not enhanced in the presence of the proteosome inhibitor MG132, suggesting that MDM2-mediated ubiquitination of Notch1 is not involved in targeting the protein for proteosomal degradation (Figure 5A). This discovery also correlates with the data presented above, suggesting that MDM2 leads to an increase in NICD1 protein levels (Figure 1). An *in vitro* ubiquitination assay was then set up which employed purified components. Ubiquitination of NICD1 was not observed in the absence of MDM2 (Figure 5B, lanes 1 and 2). However, in the presence of E1, E2 and full-length MDM2, NICD1 was subject to modification producing a characteristic ubiquitin ladder *in vitro* (Figure 5B, lanes 3–5).

Having confirmed that NICD1 undergoes MDM2-mediated ubiquitination, the domains involved in ubiquitination were investigated. MDM2 missing the N-terminal domain, (ΔN) had no effect on ubiquitination levels, compared with wild-type MDM2 (WT) (Figure 5C). However, a dramatic reduction in ubiquitination was observed when MDM2 missing the RING domain (ΔR) or AD (ΔAD), were examined (Figure 5C). This implicates the involvement of both these domains in...
Figure 3  MDM2 binds to the RAM and ANK domains of NICD1

(A) Biotin-tagged peptides of NICD1 (1–51) (3.5 μM) or DMSO control (D) were captured on a streptavidin-coated microtitre plate and incubated with purified full-length MDM2 (200 ng). Binding was detected with an anti-MDM2 (2A10) antibody, followed by chemiluminescence with enhanced chemiluminescence reagent. Peptides that bound MDM2 are listed underneath with a diagram representing domains of NICD1. NLS, nuclear localization sequence; TA, transactivation domain. (B and C) Biotin-tagged peptides of NICD1 (1–51) (3.5 μM) or DMSO (D) were captured on a streptavidin-coated microtitre plate and incubated with the isolated acid domain (MDM2-AD) (200 ng) (B) or the isolated N-terminal hydrophobic pocket (MDM2-N) (200 ng) (C). MDM2 binding was detected with anti-MDM2 (2A10 and 4B2) antibodies, followed by chemiluminescence with an enhanced chemiluminescence reagent. Experiments presented in this Figure are representative of at least three separate experiments. RLU, relative luciferase units.

MDM2-mediated ubiquitination. The RING domain and AD of MDM2 have previously been shown to be crucial for MDM2-mediated ubiquitination of other substrates [17,18,22,23].

We have previously identified a number of ligands that interact with the AD of MDM2 that are specific inhibitors of the E3 ligase activity of MDM2 [18]. Therefore, considering the results above,
we examined the MDM2 inhibitors in the in vitro ubiquitination assay to determine whether they would inhibit ubiquitination of NICD1. In a similar manner to other MDM2 substrates, such as p53 and NUMB, MDM2-mediated ubiquitination of NICD1 was disrupted in the presence of the p53-derived acid domain-binding ligand BOX-V or another acid domain-binding ligand derived from the retinoblastoma tumour suppressor protein, Rb1, but not by the MDM2 N-terminal-binding peptide BOX-I (Figure 5D), confirming the significance of the AD. Further, peptides 1, 14 and 19 derived from NICD1, that were found to bind to MDM2 (Figure 3A), were also found to inhibit MDM2-catalysed ubiquitination of NICD1, supporting the significance of these regions in the interaction with MDM2 (Figure 5E). Peptides 2, 15 and 20 did not bind to MDM2 and indeed were unable to inhibit MDM2-mediated ubiquitination (Figure 5E). In addition, similar to other AD-binding ligands, these NICD1 peptides also could inhibit MDM2-catalysed ubiquitination of p53 (Figure 5E).

The results presented above suggest that MDM2 does not appear to lead to degradation of NICD1 in cells. To support these results, mutation of Lys48 of ubiquitin to arginine did not result in a decrease in ubiquitination (Figure 5F). Furthermore, a mutant form of ubiquitin which can only form ubiquitin chains on Lys48 (K48 only) was unable to produce the characteristic ubiquitination ladder (Figure 5F). As ubiquitination on Lys48 is known to be a signal for proteasomal degradation, these results are in agreement with the observation that MDM2-mediated ubiquitination of NICD1 does not involve proteasomal degradation. These results also correlate with the data presented above demonstrating activation rather than degradation of Notch1.
Figure 5  Non-degradative ubiquitination of NICD1 by MDM2

(A) H1299 cells were transfected with NICD1 (7 μg) of His6-ubiquitin (5 μg), in the presence or absence of MDM2 (2–5 μg) as indicated. Samples were supplemented with empty vector, so that the final concentration of DNA was the same in all reactions. Cells were left untreated or treated for 5 h with 25 μM MG132 before harvesting as indicated. His6-tagged ubiquitinated proteins were isolated using Ni-NTA agarose (Qiagen) 48 h post-transfection and analysed by immunoblotting on a 3–8 % Tris-Acetate gel (Invitrogen) with anti-NICD1 antibody. Ub-NICD1, ubiquitinated NICD1. (B) NICD1 (250 ng) was incubated in the presence of ubiquitin (2 μM) and in the presence of E1 (UBE-1) (100 nM), E2 (UbcH5) (1 μM) and increasing amounts of MDM2 (50, 100 or 200 ng) as indicated. Ubiquitination was detected by immunoblotting with an anti-NICD1 antibody, where Ub-NICD1 represents ubiquitinated NICD1. (C) Upper panel, NICD1 (250 ng) was incubated in the presence of ubiquitin (2 μM), E1 (UBE-1) (100 nM), E2 (UbcH5) (1 μM) and wild-type MDM2 (WT) or MDM2 missing the AD (ΔAD), the RING domain (ΔR) or the N-terminus (ΔN) (100 ng). Ubiquitination was detected by immunoblotting with anti-NICD1 antibody, where Ub-NICD1 represents ubiquitinated NICD1. Lower panel, SDS/PAGE with Coomassie Blue staining of MDM2 wild-type and mutant proteins, the same amounts as used in the ubiquitination assay. Molecular masses are indicated to the left-hand side in kDa. (D) NICD1 (250 ng) was incubated with ubiquitin (2 μM), E1 (100 nM), E2 (1 μM) and in the presence of MDM2 (100 ng) and ligands (BOX-I, Rb1 and BOX-V) (50 μM) or DMSO control as indicated. Samples were analysed by SDS/PAGE and immunoblotting with anti-NICD1 antibody, where Ub-NICD1 represents ubiquitinated NICD1. (E) NICD1 (250 ng) or full-length p53 protein (50 ng) was incubated with ubiquitin (2 μM), E1 (100 nM), E2 (1 μM) in the presence of MDM2 (50 ng) and NICD1 peptides (NICD1 pep) (50 μM) or DMSO as indicated. Samples were analysed by immunoblotting with anti-NICD1 antibody, where Ub-NICD1 represents ubiquitinated NICD1. Experiments presented in this Figure are representative of at least three separate experiments.

NICD1 transcriptional activation is enhanced by MDM2

Following on from the observations described above, the effect of MDM2 on activation of Notch1 was examined further. Activation of the Notch signalling pathway requires that the cleaved intracellular active domain of Notch, NICD, translocates to the nucleus and binds RBP-Jκ (CSL/CFB1), where it recruits the co-activator proteins Mastermind and CBP (cAMP-response-element-binding protein-binding protein)/p300 and is converted from a transcriptional suppressor into an activator [2]. A luciferase assay using the luciferase gene under the control of the RBP-Jκ promoter in MCF7 breast cancer cells was examined. When NICD1 was co-transfected with the RBP-Jκ promoter, the promoter was stimulated (Figure 6A). In the presence of overexpressed MDM2, NICD1 activity was further enhanced (Figure 6A), correlating with the results presented above and suggesting that MDM2 activates Notch1 transcriptional activity. MDM2 transfected on its own without NICD1 had no effect on transcriptional activity (Figure 6A). When NUMB was also co-transfected with NICD1 and MDM2, it could reverse the
Figure 6  MDM2 enhances the activation of NICD1

(A) MCF7 cells were transfected with 12× RPB-Jκ Reporter (100 ng) and/or Renilla luciferase (100 ng) and Notch (400 ng) in the presence or absence of NUMB (400 ng) and MDM2 (50 ng) as indicated. DNA was equalized by addition of empty vector. Cells were analysed using the Dual Luciferase Reporter Assay (Promega) following the manufacturer’s instructions. (B) H1299 cells were transfected with 12× RPB-Jκ Reporter (10 ng) and/or Renilla luciferase (10 ng) and NICD1 (400 ng) in the presence or absence of wild-type MDM2 (Wt) or mutant MDM2 (50 ng) as indicated, where ∆R is missing the RING domain, ∆AD is missing the AD, ∆N is missing the N-terminus of MDM2 and C464A is a point mutation of MDM2. DNA was equalized by the addition of empty vector. Cells were analysed using the Dual Luciferase Reporter Assay following the manufacturer’s instructions. Bottom panel, Western blot analysis of the samples used in the Luciferase assay using anti-MDM2 and anti-NICD1 antibodies as indicated. MDM2 blots were overlaid with anti-β-actin antibody. (C and D) MCF7 cells transfected with the 12× RPB-Jκ Reporter (100 ng) and/or Renilla luciferase (100 ng) and NICD1 (400 ng), in the presence or absence of MDM2 (50 ng) as indicated. Peptides (5 and 10 μM) were added 7 h post-transfection and incubated for 24 h. Cells were analysed using the Dual Luciferase Reporter Assay following the manufacturer’s instructions. EV, empty vector; Pep7, peptide 7; RLU, relative luciferase units.

The effect of MDM2 on Notch activity (Figure 6A), supporting the evidence that they compete for binding. This effect was not p53-dependent as we also observed that MDM2 could enhance NICD1 activation in p53-null H1299 cells (Figure 6B). As H1299 cells have very low levels of endogenous MDM2, we also used these cells to examine the effect of mutants of MDM2 to determine the domains that could contribute to the stimulation of Notch1 activation (Figure 6B). Both the AD and N-terminal binding regions of MDM2 were found to be significant for the increase in NICD1 activity observed (Figure 6B). To confirm that they were expressed at similar levels in the cell, the samples were also subject to Western blotting with antibodies against MDM2, NICD1 and blots overlaid with anti-β-actin antibody (Figure 6B). All the mutant MDM2 proteins were expressed at similar or higher levels than wild-type MDM2 (Figure 6B). Interestingly, MDM2 missing the RING domain (ΔR) also demonstrated a reduction in activity compared with wild-type MDM2. Therefore a RING finger mutant of MDM2, C464A, which has no E3 ligase activity, was also examined (Figure 6B). This form of MDM2 with a point mutation in the RING finger domain also demonstrated a reduction in activation of NICD1 (Figure 6B), implying a role for ubiquitination in MDM2-mediated activation of NICD1.

We have shown that MDM2 AD inhibitors can reduce the levels of NICD1 in cells [17] and that they prevent MDM2-mediated ubiquitination of NICD1 (Figure 5D). Therefore these peptides were examined in the luciferase assay. Interestingly, the AD-binding ligands could overcome the MDM2-mediated enhancement of NICD1 activity (Figure 6C). In fact, addition
of the peptides could reduce NICD1 activity even in the presence of endogenous levels of MDM2 (Figure 6C). NUMB peptide 7 does not bind to MDM2 or NICD1 and was used as a negative control. Interestingly, the MDM2 inhibitor Nutlin was also unable to reduce NICD1 activity (Figure 6C).

**MDM2 can synergize with NICD1 to inhibit p53-dependent apoptosis**

Suppression of p53-mediated apoptotic response is thought to play an important role in Notch-induced tumorigenesis in breast cancer [11]. Indeed, transfection of the breast cancer cell line ZR75 with NICD1 resulted in a decrease in apoptosis, measured by caspase 3/7 activation (Figure 7A). This effect was observed before and after DNA damage with two different damaging agents, melphalan and cisplatin (Figures 7A and 7B). The effect of MDM2 was then examined. When MDM2 was transfected into cells on its own, inhibition of caspase activation was observed (Figure 7B). Furthermore, when cells were co-transfected with NICD1 and MDM2, we observed that MDM2 and NICD1 could synergize to inhibit activation of apoptosis before and after DNA damage in ZR75 cells (Figure 7B). However, NICD1 or MDM2 overexpression had no effect on caspase activation in the p53-null H1299 cell line (Figure 7C).
MDM2 AD-binding ligands can induce p53-dependent apoptosis in cells [17]. When tested in the presence of NICD1, we observe that the AD-binding ligands Rb1 and NUMB peptides 8 and 9 could overcome the reduction in caspase activation observed by transfection of NICD1 in two different cell lines (Figure 7D). Peptide 7 derived from NUMB was used as a negative control (Figure 7D). Nutlin is a potent inducer of apoptosis and could also overcome the effects of NICD1 in this assay (Figure 7D).

The effect of NICD1 on cell proliferation was then examined using the Cell Titer Aqueous One solution cell proliferation kit. When cells were transiently transfection with NICD1, an increase in cell proliferation was observed in both ZR75 and A375 cells (Figure 7E). MDM2 could also enhance cell proliferation in this assay (Figure 7E) and a synergistic effect on cell proliferation was observed in the presence of both MDM2 and NICD1 in ZR75 cells and a slight increase was also observed in A375 cells (Figure 7E). The effect of NICD1 and MDM2 on cell growth was also examined in colony formation assays using A375 cells (Figure 7F). NICD1 was found to increase cell growth compared with the empty vector control. Transfection of the cells with MDM2 also resulted in an increase in colonies and a synergistic increase in cell growth was observed in the presence of both NICD1 and MDM2. In addition, the colonies appeared larger (Figure 7F). However, at high expression levels of MDM2, we observed that MDM2 actually caused a growth arrest, which we have previously observed in many other cell lines, suggesting that the effects of MDM2 are dose-dependent.

**DISCUSSION**

Notch signalling plays a crucial role in development and adult homeostasis and is frequently found deregulated in human disease, underscoring the importance of understanding the regulation of the Notch pathway. Notch has varied roles in cancer, which probably reflect its multiple functions in development and tissue homeostasis. Notch1 has been shown to have an oncogenic role in cancers such as breast, T-ALL and melanoma. However, Notch1 exerts an anti-oncogenic role in squamous cell carcinomas of the skin, prostate and small cell lung cancer. The regulation of Notch to cause it to act as an oncogene or a tumour suppressor is not clearly understood, however it appears that cellular context may determine the outcome.

Ubiquitination and degradation of the Notch regulator NUMB in breast cancer leads to aberrant activation of Notch [10,11]. The E3 ligase responsible for the degradation of NUMB in breast cancer is unknown, although MDM2 is frequently found overexpressed in breast cancer and overexpression of MDM2 in transgenic mice null for p53 resulted in mammary tumorigenesis [24]. In the present study we show that, in the presence of overexpressed MDM2, higher levels of activated Notch1 are found overexpressed MDM2, RING domain of MDM2 was shown to be crucial for the ubiquitination of NICD1. Deletion of the RING domain of MDM2 has no effect on MDM2-catalysed ubiquitination of NICD1. Notch 1 activity is positively regulated by ubiquitin. Previous reports have suggested that the Notch4 receptor is ubiquitinated and degraded by Lys48-linked ubiquitin chains, whereas Lys48-linked polyubiquitin chains have a regulatory function not involved in degradation. The roles of unconventional polyubiquitin chains linked through Lys6, Lys11, Lys27, Lys29 or Lys33 are not yet well understood. These non-canonical linkages are abundant in vivo and they may also target proteins for degradation [33]. However, recent evidence suggests a function for ubiquitination in activating transcription factors [34]. Indeed, MDM2 interacts with and ubiquitinates TAT, resulting in transcriptional activation [35]. Mutation of Lys48 had no effect on MDM2-catalysed ubiquitination of NICD1. Notch 1 is another example in a growing list of transcription factors whose activity is positively regulated by ubiquitin. Previous reports have suggested that the Notch4 receptor is ubiquitinated and degraded by MDM2 [36]. The function of each isoform appears to be tissue- and context-dependent [37]. However, in which processes the different Notch receptors contain specific or redundant functions still needs to be determined. Also it is possible that heterotypic NICD 1–4 interactions could occur, which may have a biological outcome. In addition, ubiquitination of the same substrate could have seemingly opposite effects depending on the nature of the modification and further studies are required to address these questions.

The interaction between NUMB and the intracellular domain of Notch prevents its nuclear localization and signalling, and an intact PTB domain of NUMB is required for this function. The PTB domain of NUMB is also involved in forming the interaction with MDM2 (amino acids 113–148) [17]. Our results

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MDM2 positively modulates the Notch signalling pathway by a number of mechanisms: (1) ubiquitinating and degrading the Notch regulator NUMB, preventing NUMB from regulating Notch1 levels in the cell; (2) disrupting the NUMB–Notch complex and allowing unrestricted Notch1 activity; and (3) directly interacting with NICD1, resulting in ubiquitination and activation of the Notch 1 signalling pathway. Ub, ubiquitin.

Figure 8  Model of the regulation of the Notch signalling pathway by MDM2

Inhibitors of the Notch signalling pathway, such as GSIs (γ-secretase inhibitors), are currently in clinical trials for the treatment of cancers such as melanoma and T-ALL. However, they do not specifically target the Notch pathway and have been shown to cause some undesirable side effects. In addition, many patients develop resistance to GSIs [38–40]. Regulation of E3 ligases is potentially one significant strategy for controlling the activity of the Notch pathway. Targeting the AD of MDM2 using small molecule peptide inhibitors resulted in stabilization of p53 and NUMB levels and a reduction in the levels of the activated intracellular domain of Notch1 [17]. In addition, Notch1 transcriptional activity was reduced in the presence of AD-binding ligands. Therefore, in patients with overexpressed MDM2, it may be useful to develop drugs that target the acid domain to prevent aberrant activation of Notch1.

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