F-Box Protein FBXO31 Mediates Cyclin D1 Degradation to Induce G1 Arrest Following DNA Damage

Manas K. Santra, Narendra Wajapeyee, and Michael R. Green

Howard Hughes Medical Institute, Programs in Gene Function and Expression and Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA

Abstract

In response to DNA damage eukaryotic cells initiate a complex signalling pathway, termed the DNA damage response (DDR), which coordinates cell cycle arrest with DNA repair. Studies have shown that oncogene-induced senescence, which provides a barrier to tumour development, involves activation of the DDR1–3. Using a genome-wide RNA interference (RNAi) screen, we have identified 17 factors required for oncogenic BRAF to induce senescence in primary fibroblasts and melanocytes⁴. One of these factors is an F-box protein, FBXO31, a candidate tumour suppressor encoded in 16q24.3, a region in which there is loss of heterozygosity in breast, ovarian, hepatocellular and prostate cancers^{5–9}. Here we study the cellular role of FBXO31, identify its target substrate and elucidate the basis for its growth inhibitory activity. We show that ectopic expression of FBXO31 acts through a proteasome-directed pathway to mediate degradation of cyclin D1, an important regulator of G1 to S phase progression, resulting in G1 arrest. Cyclin D1 degradation results from a direct interaction with FBXO31 and is dependent upon the F-box motif of FBXO31 and phosphorylation of cyclin D1 at threonine-286, which is known to be required for cyclin D1 proteolysis. The involvement of the DDR in oncogene-induced senescence prompted us to investigate the role of FBXO31 in DNA repair. We find that DNA damage induced by γ -irradiation results in increased FBXO31 levels, which requires phosphorylation of FBXO31 by the DDR-initiating kinase, ATM. Significantly, RNAi-mediated knockdown of FBXO31 prevents cells from undergoing efficient G1 arrest following γ -irradiation and dramatically increases sensitivity to DNA damage. Finally, we show that diverse DNA damaging agents all result in a large increase in FBXO31 levels, indicating that induction of FBXO31 is a general response to genotoxic stress. Our results reveal FBXO31 as a regulator of the G1/S transition that is specifically required for DNA damage-induced growth arrest.

We studied FBXO31 function in human SK-MEL-28 melanoma cells. We first tested whether, as in breast cancer cell lines⁹, ectopic expression of FBXO31 could induce G1 arrest in SK-MEL-28 cells. Fluorescence-activated cell sorting (FACS) analysis revealed, as

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Correspondence and requests for materials should be addressed to M.R.G. (michael.green@umassmed.edu).

Author Contributions M.K.S. and N.W. contributed equally to this work. M.K.S., N.W. and M.R.G. conceived and designed the experiments. M.K.S. and N.W. performed the experiments and, together with M.R.G., analyzed the data and wrote the paper.

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expected, that nocodazole treatment of SK-MEL-28 cells resulted in G2/M arrest (Fig. 1a). Ectopic expression of FBXO31 prevented the nocodazole-induced G2/M block and instead substantially increased the fraction of cells in G1. Identical results were obtained in cells ectopically expressing FBXO31 in the absence of nocodazole (Supplementary Fig. 1). Moreover, ectopic expression of FBXO31 blocked DNA synthesis (Fig. 1b). Notably, ectopic expression of FBXO31 also markedly inhibited the growth of SK-MEL-28 cells in culture (Supplementary Fig. 2a) and SK-MEL-28 mouse xenografts (Supplementary Fig. 2b).

F-box proteins are best known for their role as the substrate-recognition components of the SCF (SKP/Cullin/F-box protein) class of E3 ubiquitin ligases¹⁰. To investigate the basis by which FBXO31 induces G1 arrest, we analyzed a panel of cell cycle regulatory proteins by immunoblot analysis following ectopic expression of FBXO31. Figure 1c shows that following expression of FBXO31, but not vector alone (Supplementary Fig. 3), the level of cyclin D1, a key regulator of the G1/S phase transition¹¹, markedly declined. By contrast, the levels of other G1 cyclins and the G2/M regulator cyclin B1 were unaffected. Ectopic FBXO31 expression also had no effect on the levels of all cyclin-dependent kinases (CDKs) (Fig. 1d) and CDK inhibitors (Fig. 1e) examined. As expected, the reduction in cyclin D1 levels was accompanied by an increase in the fraction of cells in G1 (Supplementary Fig. 4). RNAi-mediated knockdown of cyclin D1 in SK-MEL-28 cells resulted in a similar G1 arrest (Supplementary Fig. 5). Collectively, these results suggest that FBXO31 induces G1 arrest through selective degradation of cyclin D1.

We next sought to determine whether the FBXO31-mediated decrease in cyclin D1 resulted from proteasomal degradation. Figure 2a shows that addition of the proteasome inhibitor lactacystin blocked the ability of ectopically expressed FBXO31 to decrease cyclin D1 levels. Consistent with this result, cyclin D1 mRNA levels were unaffected by ectopic FBXO31 expression (Fig. 2b). Moreover, following ectopic expression of FBXO31 the half-life of cyclin D1 was substantially decreased (Supplementary Fig. 6).

Proteasomal-mediated degradation of cyclin D1 requires phosphorylation on threonine-286^{12–14}. We therefore tested whether FBXO31-mediated degradation of cyclin D1 was dependent upon threonine-286 phosphorylation. SK-MEL-28 cells were stably transfected with a plasmid expressing HA-tagged cyclin D1 or a cyclin D1 derivative bearing a threonine-to-alanine substitution at position 286 [cyclin D1(T286A)]¹² and then transduced with a retrovirus expressing FBXO31. Figure 2c shows that ectopic expression of FBXO31 resulted in degradation of wild type cyclin D1 but not the cyclin D1(T286A) mutant. Importantly, following expression of cyclin D1(T286A), ectopically expressed FBXO31 failed to induce efficient G1 arrest (Fig. 2d and Supplementary Fig. 7a) or block DNA synthesis (Fig. 2e).

To test whether FBXO31 and cyclin D1 interact, we performed co-immunoprecipitation experiments. SK-MEL-28 cells transduced with a retrovirus expressing myc-tagged FBXO31 were stably transfected with a plasmid expressing HA-tagged cyclin D1 and treated with lactacystin to prevent cyclin D1 degradation (see Fig. 2a). The results of Fig. 3a show the presence of cyclin D1 in the FBXO31 immunoprecipitate, which was most evident

when lactacystin was added. A similar result was obtained in the reciprocal co-immunoprecipitation. By contrast, FBXO31 failed to interact with cyclin D1(T286A) (Supplementary Fig. 7b). Figure 3a also shows, consistent with previous reports⁹, that FBXO31 was part of the SCF complex as evidenced by its association with SKP1 and CUL1. The co-immunoprecipitation experiment of Fig. 3b shows that there was also an interaction between the endogenous FBXO31 and cyclin D1 proteins.

To determine whether the F-box motif of FBXO31 was required for cyclin D1 proteolysis, we used an FBXO31 derivative in which the F-box had been deleted (FBXO31^{ΔF}). In contrast to wild type FBXO31, ectopic expression of FBXO31^{ΔF} did not result in decreased levels of cyclin D1 (Fig. 3c). Consistent with this finding, ectopic expression of FBXO31 but not FBXO31^{ΔF} resulted in polyubiquitination of cyclin D1 (Fig. 3d). As expected, cyclin D1(T286A) was not polyubiquitinated. Figure 3e shows that FBXO31 directed polyubiquitination of cyclin D1 *in vitro* in the absence of any other F-box protein.

As noted above, it has been previously shown that oncogene-induced senescence involves activation of the DDR1–3. We therefore considered the possibility that in addition to its role in BRAF-induced senescence⁴, FBXO31 might also be involved in mediating G1 arrest following DNA damage. Figure 4a shows that following induction of DNA damage by γ -irradiation, the levels of FBXO31 increased and, consistent with previous reports¹⁵, this was accompanied by a decrease in cyclin D1 levels. Quantitative real-time RT-PCR confirmed that following γ -irradiation, FBXO31 and cyclin D1 mRNA levels were unchanged (Supplementary Fig. 8). Consistent with this finding, in untreated SK-MEL-28 cells the low levels of FBXO31 resulted, at least in part, from proteasomal degradation (Supplementary Fig. 9). Significantly, RNAi-mediated knockdown of FBXO31 prevented the decrease in cyclin D1 following γ -irradiation (Fig. 4b and Supplementary Fig. 10). In support of this conclusion, γ -irradiation decreased the half-life of cyclin D1 in SK-MEL-28 cells expressing a control non-silencing (NS) shRNA but not an FBXO31 shRNA (Supplementary Fig. 11). Most importantly, in FBXO31 knockdown (KD) SK-MEL-28 cells γ -irradiation failed to induce G1 arrest (Fig. 4c and Supplementary Fig. 12).

Phosphorylation of cyclin D1 at threonine-286 can be mediated by glycogen synthase kinase 3 β (GSK3 β)^{12,13} and through a MAP kinase pathway¹⁴. We found that GSK3 β was not required for cyclin D1 degradation following γ -irradiation, whereas blocking MAP kinase signalling using a chemical inhibitor of MEK prevented cyclin D1 degradation following γ -irradiation (Supplementary Fig. 13).

ATM is a serine-threonine protein kinase that plays a critical role in the DDR^{16–18}. In response to DNA double-strand breaks, ATM is autophosphorylated on several residues including serine-1981, and functions by phosphorylating, and in many cases stabilizing, a number of downstream protein targets that function in the DDR^{16,19}. FBXO31 contains two putative ATM phosphorylation sites at amino acids 278/279 and 400/401, the former of which is conserved (Fig. 4d, top). Co-immunoprecipitation experiments revealed that FBXO31 and ATM interacted (Supplementary Fig. 14). A GST-fusion protein containing the 278/279serine-glutamine site was phosphorylated by ATM *in vitro* (Fig. 4d, bottom), and an FBXO31 derivative bearing a serine-to-alanine mutation at position 278 failed to

accumulate following γ -irradiation (Fig. 4e) or upon co-transfection with ATM (Fig. 4f). In ATM KD SK-MEL-28 cells, FBXO31 was not induced following γ -irradiation and, accordingly, cyclin D1 levels remained unchanged (Fig. 4g and Supplementary Fig. 15). Significantly, knockdown of FBXO31 substantially increased sensitivity of SK-MEL-28 cells to γ -irradiation (Fig. 4h). Collectively, these results indicate that following γ -irradiation ATM directly phosphorylates FBXO31, which results in FBXO31 stabilization and subsequent cyclin D1 degradation.

Finally, we tested whether other DNA damaging agents also induced FBXO31. Figure 4i shows that FBXO31 levels greatly increased and cyclin D1 levels declined to varying extents following UV irradiation, X-ray irradiation, oxidative stress (H_2O_2), or addition of the chemotherapeutic DNA damaging agents etoposide, adriamycin, cisplatin or fluorouracil. Based upon these results we conclude that induction of FBXO31 is a general response to genotoxic stress.

Collectively, the results reported here show that FBXO31 is induced by DNA damage and, once stabilized, targets cyclin D1 for degradation by ubiquitin-mediated proteolysis, leading to G1 arrest (Fig. 4j). Our results address a long-standing problem in the DNA repair field. It has been previously shown that G1 arrest following DNA damage occurs in a two-phase response, referred to as initiation and maintenance¹⁵. The maintenance phase is a slow response that is primarily due to p53-dependent transcription induction of the cell cycle inhibitor p21. By contrast, initiation is a rapid, p53-independent response that primarily results from cyclin D1 degradation. However, the mechanistic basis by which cyclin D1 becomes rapidly degraded after genotoxic stress remained to be determined. Our results indicate that initiation of G1 arrest following genotoxic stress is due to induction of FBXO31, which then interacts with cyclin D1 and mediates its degradation.

Two other F-box proteins, FBXO4 (also called FBX4) and FBXW8, have been previously reported to mediate cyclin D1 degradation^{14,20}. FBXO31, FBXO4 and FBXW8 likely function through a common mechanism as evidenced by the observations that all three proteins are incorporated into the SCF complex and require phosphorylation of cyclin D1 at threonine-286 to direct cyclin D1 degradation. However, an important distinction between the three proteins is that FBXO4 and FBXW8 appear to be required for normal cell cycle progression^{14,20}, whereas we find no evidence for a cell cycle defect or decreased growth rate following knockdown of FBXO31 (ref. 4; see above and data not shown). Moreover, in contrast to FBXO31, the levels of FBXO4 and FBXW8 do not increase following γ -irradiation (Supplementary Figure 16a), and knockdown of FBXO4 or FBXW8 does not affect cyclin D1 degradation in γ -irradiated SK-MEL-28 cells (Supplementary Figure 16b). The results presented here reveal that FBXO31 is a dedicated checkpoint protein whose function is to arrest cells following genotoxic stress.

METHODS SUMMARY

Cell cycle analysis

For FACS analysis, SK-MEL-28 cells transduced with retrovirus (MOI=20) expressing FBXO31 or empty vector, in the presence or absence of a plasmid expressing cyclin D1 or

cyclin D1(T286A), were collected 48 h after virus infection, after having been treated with 8 $\mu\text{g ml}^{-1}$ of nocodazole (Sigma) for the final 16 h. Cells were washed with cold phosphate-buffered saline (PBS) and fixed with ethanol. Nocodazole treatment for γ -irradiation-treated cells was performed as previously described¹⁵ following treatment of SK-MEL-28 cells with a non-silencing or FBXO31 shRNA. FACS samples were analyzed by the FACSCalibur system (BD Biosciences).

For BrdU labeling, SK-MEL-28 cells transduced with retrovirus expressing FBXO31 or empty vector, in the presence or absence of a plasmid expressing cyclin D1 or cyclin D1(T286A), were collected 48 h after virus infection, after having been treated with BrdU (20 μM) for the final 4 h. Cells were then harvested, fixed in 70% ethanol and stained sequentially with an α -BrdU mouse antibody (Calbiochem), FITC-conjugated goat anti-mouse antibody (Calbiochem) and 4',6-diamidino-2-phenylindole (DAPI) (Sigma). DAPI-stained cells were scored as BrdU-positive or -negative using a Zeiss Axiophot 2 fluorescence microscope; approximately 500–600 cells per sample were scored.

Ubiquitination assays

For *in vivo* assays, SK-MEL-28 cells were co-transfected with plasmids expressing HA-cyclin D1 or HA-cyclin D1(T286A) and Flag-tagged ubiquitin and then transduced with an FBXO31 or FBXO31^F retrovirus. Polyubiquitinated cyclin D1 was detected by immunoprecipitation of ubiquitin with an anti-Flag antibody followed by immunoblotting with an anti-HA antibody for cyclin D1. For *in vitro* assays, 293T cells were transfected with vectors encoding myc-FBXO31, -CUL1, -SKP1 and -ROC1. The SCF^{FBXO31} (E3) complexes were immunopurified from the cell lysate using an α -myc antibody and incubated with *in vitro*-translated GST-tagged cyclin D1 in the presence of purified, recombinant active Erk2, E1, E2 (UbcH5A), ATP and ubiquitin.

METHODS

Cell lines and culture

Human melanoma SK-MEL-28 cells were obtained from ATCC and grown as recommended. SK-MEL-28 cell lines expressing myc-FBXO31 or myc-FBXO31^F were generated by retroviral transduction. Briefly, SK-MEL-28 cells were seeded (1×10^6 cells/10 cm dish) and the next day transduced with 10 ml FBXO31- or FBXO31^F-expressing retrovirus (MOI = 20) using 10 $\mu\text{g ml}^{-1}$ polybrene (Sigma). The medium was changed 12 h after virus infection.

Plasmids

Retroviral vectors expressing myc-FBXO31 and myc-FBXO31^F were previously described⁹ and provided by Raman Kumar (University of Adelaide, Australia). Plasmids expressing HA-cyclin D1 and HA-cyclin D1(T286A) were previously described²¹ and purchased from Addgene (Addgene plasmids 11181 and 11182, respectively). Other plasmids were obtained as follows: Flag-ubiquitin (Simon Cook, Babraham Institute, UK), Flag-FBXW6 (Michele Pagano, New York University School of Medicine), V5-FBXW8

(Osamu Tetsu, University of California, San Francisco), and wild type and kinase-dead Flag-ATM (Michael Kastan, St. Jude Children's Research Hospital).

Immunoblot analysis

Protein extracts were prepared by lysis in a buffer containing (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na₃VO₄) and protease inhibitors in the presence or absence of a phosphatase inhibitor cocktail (Sigma), as needed. Blots were probed with the following antibodies: α -human cyclin D1 mouse monoclonal (BD Bioscience Pharmingen), α -phospho cyclin D1(T286) rabbit polyclonal (Cell Signaling), α -cyclin D2 mouse monoclonal (Abcam), α -cyclin D3 mouse monoclonal (Santa Cruz), α -cyclin B1 mouse monoclonal (Upstate), α -cyclin E2 rabbit monoclonal (Abcam); α -CDK1 mouse monoclonal (BD Bioscience Pharmingen), α -CDK2 rabbit polyclonal (Abcam), α -CDK4 rabbit polyclonal (Santa Cruz), α -CDK6 rabbit polyclonal (Santa Cruz), α -p14 rabbit polyclonal (Santa Cruz), α -p16 rabbit polyclonal (Cell Signaling), α -p27 mouse monoclonal (Santa Cruz) and α -p57 mouse monoclonal (Sigma), α -tubulin mouse monoclonal (Sigma), α -HA mouse monoclonal (Roche), α -myc mouse monoclonal (Roche), α -Flag mouse monoclonal (Sigma), α -SKP1 mouse monoclonal (BD Bioscience Pharmingen), α -CUL1 rabbit polyclonal (Cell Signaling), α -actin mouse monoclonal (Sigma), α -ATM rabbit monoclonal (Cell Signaling), α -ATM-P(S1981) mouse monoclonal (Cell Signaling), α -GSK3 β mouse monoclonal (BD Transduction Laboratories), α -p44/42 (Erk1/2) rabbit polyclonal (Cell Signaling), α -phospho-p44/42 (Erk1/2) rabbit monoclonal (Cell Signaling), α -FBXO4 rabbit monoclonal (Rockland Immunochemicals), α -FBXW8 rabbit polyclonal (Saier Biotechnology Inc) and α -PARP1 mouse monoclonal (EMD Biosciences, Inc). To raise the rabbit polyclonal α -FBXO31 antibody, a mixture of two peptides (EGRGRQGPRESQPSAQPRAC and CPVGVSSRNEDYPRT) was used to immunize rabbits (AnaSpec) followed by affinity purification. The antibody was used to detect endogenous FBXO31 by immunoprecipitation-immunoblot analysis. Where indicated, 5 μ M of lactacystin (Calbiochem) was added for 8 h prior to protein extract preparation.

Quantitative real-time RT-PCR

Total RNA was isolated and reverse transcription was performed as described²², followed by quantitative real-time PCR using Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen). Primer sequences are as follows: cyclin D1, forward (5'-TCTACACCGACAACCTCCATCC-3') and reverse (5'-TTCCACTTGAGCTTGTTCCACC-3'); FBXO31, forward (5'-AATCCGGCCTTTTGACCAGA-3') and reverse (5'-TCCGCTCACAGGAAGAGCAC-3').

RNA interference

FBXO31 and ATM knockdown cell lines were generated by stable transduction of 6×10^4 SK-MEL-28 cells with the following shRNAs from Open Biosystems: FBXO31 (source location V2HS_157523, clone location SH2232-d-1 and source location V2HS_25023, clone location SH2497-b-9), ATM (source location V2HS-89368, clone location SH2178-a-12 and source location V2HS-192800, clone location SH116-a-9), FBXO4 (source location V2HS-15758, clone location SH2245-b-3), GSK3 β (source location V2HS-114292,

clone location SH2049-a-7) and non-silencing (5'-TCTCGCTTGGGCGAGAGTAAG-3'). SiRNAs were purchased from Sigma and their sequences (sense strand) are as follows: FBXO31 (5'-CUGAUGAAGU UCAUCUACA UU-3'), FBXW8 (5'-AAGAUGUGCACAGGUGAGCAA-3'), FBXO4 (5'-CAAUGAAGGUGAUGAUCAA UU-3'), cyclin D1 (5'-CUGUCCACUCCUACG AUA UU-3' and 5'-CUGUGUAUCUCUUUCACA UU-3') and Luciferase (5'-UGAU CAAAUACAAGGGAUA-3').

Generation of FBXO31 mutants

The plasmid pLNCX2-FBXO319 was used as a template to generate FBXO31 mutants using the QuikChange XL site-directed mutagenesis kit (Stratagene). To generate in-frame GST-tagged fusion proteins, the wild type and mutant FBXO31 sequences were PCR amplified (using primers WT1_forward 5'-CGGGATCCGCTGCCACAGTGGAGTGC-3' and WT1_reverse 5'-GGAATTCGATGAGGTCGTCGGGGCG-3', or WT2_forward 5'-CGGAATTCCGG ATCCAGCTGCCCGAC-3' and WT2_reverse 5'-ACGTCGACCACGGGCAGCACG AACGG-3'), digested with BamH1 and EcoR1, and cloned into pGEX-4T-1 (Amersham). All constructs were confirmed by sequencing.

ATM kinase assays

In vitro ATM kinase assays were performed as described previously²³. Briefly, 293T cells were transfected with plasmids (10 µg) expressing Flag-tagged ATM or the kinase-dead mutant (ATM-KD). Cell lysates were prepared by resuspension in modified TGN buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture from Roche Molecular Biochemicals). Cell lysates were incubated with anti-Flag M2 antibody (Sigma) and protein A/G-agarose over night at 4 °C. The precipitated beads were washed with TGN buffer followed by TGN buffer plus 0.5 M LiCl, and two washes with kinase buffer (20 mM HEPES at pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 10 mM MnCl₂). Finally, the pellet was resuspended in 50 µl kinase buffer containing 10 µCi [γ -³²P]ATP and 1 µg GST-fusion substrate (described above). The kinase reaction was incubated for 20 min at 30°C and stopped by the addition of SDS-PAGE protein sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography.

Colony formation assays

5×10⁵ SK-MEL-28 cells stably expressing a NS or FBXO31 shRNA were plated in 60 mm dishes and γ -irradiated at a dose of 10 Gy. Cells were puromycin selected for 14 days, then stained with crystal violet and photographed.

Induction of DNA damage

SK-MEL-28 cells were grown as a subconfluent monolayer and treated with either γ (20 Gy), UV (50 mJ/cm²), or X-ray (20 Gy) irradiation and then incubated at 37°C prior to protein extract preparation. For hydrogen peroxide treatment, cells were treated with 0.02% H₂O₂ for 4 h. For chemotherapeutic DNA damaging agents, cells were treated with 2 µM

etoposide (Sigma), 0.2 $\mu\text{g ml}^{-1}$ doxorubicin (Sigma), 2 $\mu\text{g ml}^{-1}$ cisplatin (Sigma), 50 μM fluorouracil or, as a control, DMSO for 48 h prior to protein extract preparation.

Cell cycle analysis by two-colour FACS

Cells transduced with a retrovirus expressing FBXO31 or empty vector in the presence or absence of plasmids expressing cyclin D1 or cyclin D1(T286A), or cells stably expressing luciferase or cyclin D1 siRNAs, were incubated with BrdU (20 μM) for 4 h, fixed in 70% ethanol and stained sequentially with an α -BrdU mouse antibody (Calbiochem), Alexa 488 conjugated goat α -mouse antibody (Invitrogen) and propidium iodide (Sigma). FACS samples were analyzed by the FACSCalibur system (BD Biosciences).

Supplementary Material

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Figure 1.

Ectopic expression of FBXO31 induces G1 arrest and selective degradation of cyclin D1. **a**, FACS analysis in SK-MEL-28 cells transduced with a retrovirus expressing empty vector or FBXO31 in the absence or presence of nocodazole. **b**, DNA replication assay, monitored by bromodeoxyuridine (BrdU) incorporation (error bars, s.d., n=3). **c–e**, Immunoblot analysis showing levels of cyclins (**c**), CDKs (**d**) and CDK inhibitors (**e**) at various time points following transduction with an FBXO31 retrovirus. Tubulin was monitored as a loading control. Ectopic expression of FBXO31 did not induce a DDR (Supplementary Figure 17).

Figure 2.

FBXO31-mediated cyclin D1 degradation occurs through the proteasomal pathway. **a**, Cyclin D1 levels in SK-MEL-28 cells transduced with a retrovirus expressing FBXO31 or empty vector and treated in the presence or absence of lactacystin. **b**, Quantitative real-time RT-PCR monitoring *cyclin D1* mRNA levels (error bars, s.d., n=3). **c**, Cyclin D1 levels in SK-MEL-28 cells stably transfected with a plasmid expressing either HA-tagged cyclin D1 or cyclin D1(T286A) and transduced with an FBXO31 retrovirus. **d**, FACS analysis in cells described in (d). **e**, DNA replication assay (error bars, s.d., n=3).

Figure 3.

FBXO31 interacts with and directs ubiquitination of cyclin D1. **a**, Co-immunoprecipitation of FBXO31 with cyclin D1 and SCF complex components. The FBXO31-cyclin D1 interaction was specific (Supplementary Fig. 18) and predominantly cytoplasmic (Supplementary Fig. 19). **b**, Co-immunoprecipitation of endogenous FBXO31 and cyclin D1. **c**, Cyclin D1 levels in SK-MEL-28 cells ectopically expressing vector, FBXO31 or FBXO31 F. **d**, *In vivo* ubiquitination assay. Polyubiquitinated cyclin D1 was detected by immunoprecipitation of Flag-tagged ubiquitin followed by immunoblotting for HA-cyclin D1. **e**, *In vitro* ubiquitination assay. Immunopurified SCF complexes were incubated with GST-cyclin D1, Erk2, E1, E2, ATP and ubiquitin.

Figure 4.

Cell cycle arrest following DNA damage requires ATM-mediated induction of FBXO31. **a**, Immunoblot analysis of FBXO31 following γ -irradiation. For comparison to ectopically expressed FBXO31 levels see Supplementary Fig. 20. **b**, Cyclin D1 levels in cells expressing a NS or FBXO31 shRNA following γ -irradiation. **c**, FACS analysis. **d**, (Top) Putative ATM sites in FBXO31. (Bottom) *In vitro* phosphorylation of GST-FBXO31 fusion proteins harbouring wild type (WT) or mutated (SDM) SQ sites. ATM-WT, wild type ATM; ATM-KD, kinase dead ATM mutant. **e-f**, Levels of ectopically expressed WT or mutant FBXO31 following γ -irradiation (**e**) or ectopic ATM expression (**f**). **g**, FBXO31, cyclin D1, ATM and phosphorylated ATM in γ -irradiated cells expressing a NS or ATM shRNA. **h**, Colony formation assay in untreated or γ -irradiated cells. **i**, FBXO31 and cyclin D1 levels following treatment with DNA damaging agents. **j**, Model.