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Report

The E3 Ubiquitin Ligase EDD Regulates S-Phase and G_2/M DNA Damage Checkpoints

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KEY WORDS

EDD, UBR5, HECT, E2F1, ubiquitin ligase, CHK2, DNA damage

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ABSTRACT

The cellular response to DNA damage is critical for maintenance of genomic integrity and inhibition of tumorigenesis. Mutations or aberrant expression of the E3 ubiquitin ligase EDD have been observed in a number of carcinomas and we recently reported that EDD modulates activity of the DNA damage checkpoint kinase, CHK2. Here, we demonstrate that EDD is necessary for G_1/S and intra S phase DNA damage checkpoint activation and for the maintenance of G_2/M arrest after double strand DNA breaks. Defective checkpoint activation in EDD-depleted cells led to radio-resistant DNA synthesis, premature entry into mitosis, accumulation of polyploid cells, and cell death via mitotic catastrophe. In addition to decreased CHK2 activation in EDD-depleted cells, the expression of several key cell cycle mediators including Cdc25A/C and E2F1 was altered, suggesting that these checkpoint defects may be both CHK2-dependent and -independent. These data support a role for EDD in the maintenance of genomic stability, emphasising the potential importance of dysregulated EDD expression and/or function in the evolution of cancer.

INTRODUCTION

The DNA damage checkpoints are essential surveillance mechanisms for the maintenance of genomic integrity under conditions of genotoxic stress. Activation of the ATM-CHK2/ATR-CHK1 kinase signalling networks induces cell cycle arrest in G_1, S or G_2 phases, DNA repair and/or cell death to prevent replication of damaged DNA.[1,2] Deregulated cell cycle control is a characteristic of cancer, and compromised checkpoint function in tumour cells may be the basis of susceptibility to genotoxic chemotherapeutic agents. A fundamental aspect of checkpoint control is modulation of various key regulators e.g., cyclins, CDKs, phosphatases and transcription factors, via ubiquitin-mediated proteolysis. Deregulation of the ubiquitin-proteosome system, particularly at the level of E3 ubiquitin ligases (such as the SCF and APC/C complexes) contributes to uncontrolled proliferation and genomic instability associated with tumourigenesis.[3]

Aberrant expression of the HECT E3 ubiquitin ligase, EDD (EDD1, hHyd, UBR5), is observed in many carcinomas, particularly those of the breast and ovary, and truncating mutations are common in gastric and colon cancers with microsatellite-instability.[4-6] There is now substantial evidence that EDD is involved in the regulation of cell proliferation and tumorigenesis. Mutagenesis studies show a critical role for the Drosophila homologue of EDD (*hyd*) in the control of cell proliferation[7] and $Edd^{\Delta/\Delta}$ mice die at mid-gestation due to failed yolk sac angiogenesis and defective placental development, leading to general failure of embryonic cell proliferation and widespread apoptosis.[8]

Several lines of evidence support a role for EDD in DNA damage signalling; EDD binds the DNA-dependent protein kinase-interacting protein CIB1[9] and modulates activity of CHK2,[10] an established mediator of G_1/S, intra-S, and G_2/M phase checkpoints.[11] EDD also interacts with the human MutL homologues PMS1 and PMS2 during mismatch repair,[12] and can mediate the ubiquitinylation of topoisomerase IIβ-binding protein (TopBP1) in vitro.[13] Further, EDD depletion by siRNA in HeLa cells leads to increased sensitivity to the DNA damaging agent phleomycin,[10] suggesting a link between the reported aberrant expression or mutation of EDD in cancer and its function in the DNA damage response. Here we report a role for EDD in specific DNA damage checkpoints.

MATERIALS AND METHODS

Plasmids, siRNA Sequences and Antibodies. The sequences of the siRNA oligoribonucleotide sense strand used for the silencing of EDD and CHK2 expression respectively were (EDD1) 5`-GCA GUG UUC CUG CCU UCU UdTdT-3` and (EDD2) 5`-GCG ACU CUC CAU GGU UUC UdTdT-3`, (CHK2)[14] 5`-GAA CCU GAG GAC CAA GAA dTdT-3`. siRNA directed against Green Fluorescent Protein (GFP),[15] 5`-CUG GAG UUG UCC CAA UUC UdTdT-3` was used as a negative control. Annealed, HPLC and PAGE purified siRNA oligonucleotides were obtained from Ambion Inc. (Austin, TX). Antibodies used for immunoblotting were against EDD,[16] CHK2 (N17, Santa Cruz), phospho-CHK2(Thr68) (Santa Cruz), E2F1 (Santa Cruz, C20), Cdc25A (Neo-Markers), Cdc25C (Santa Cruz, H-6), β-actin (Sigma, AC-15). Secondary antibodies linked to horseradish peroxidase were obtained from Amersham Bioscience, UK. TOPRO-3 (Molecular Probes) was used for immunofluorescent nuclear staining and FITC-phalloidin (Sigma) for cytoplasmic staining. Antibodies used for flow cytometry analysis were FITC-anti BrdU (Chemicon International MAB3262F), anti-Phospho-Histone-3 (Upstate Rabbit polyclonal #06-570). Secondary antibodies linked to Cy2 were obtained from Jackson Immunoresearch Laboratories.

Cell Lysate Preparation. For extracts of total cellular protein, cells were harvested either by trypsinisation or scraping, and prepared in normal lysis buffer (NLB: 1% v/v Triton X-100, 50 mM HEPES pH 7.5, 150 mM NaCl, 10% v/v glycerol, 1.5 mM MgCl2, 1 mM EGTA, 10 mM Nappi, 20 mM NaF) containing an EDTA-free protease inhibitor cocktail (Roche, Germany), 0.2 mM sodium orthovanadate, 1 mM DTT and 20 μM MG-132. Western blot analyses were performed according to standard protocols.

Cell Culture, Transfection and Induction of DNA Damage. HeLa cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Vic, Australia). For siRNA transfection, cells were seeded approximately 16 h prior to transfection at a density of 1.3 x 10^4 cells.cm^{-2} and transfected with 2.8 pmol.cm^{-2} of siRNA oligoribonucleotides using opti-MEM and Oligofectamine or Lipofectamine (Invitrogen Life Technologies). When using Oligofectamine, cells were transfected in serum-free medium, then transferred into 10% FBS/DMEM 4 h later. When using Lipofectamine, cells were transfected in culture medium containing 5% FBS. To inflict DNA damage, adherent cells were treated with 65 or 130 μM (unless otherwise stated) phleomycin (InvivoGen, CA) in culture medium containing 5% fetal bovine serum for 1-3 h. Cells were then left to recover in fresh medium for the times indicated prior to harvesting or fixing.

Flow Cytometry. Forty eight hours after transfection, HeLa cells were harvested and reseeded at 1 x 10^6 cells per 59 cm^2 in culture medium containing 5% FBS. At 72 h post-transfection, cells were treated with 130 μM phleomycin for 3 h and allowed to recover in fresh medium containing 5% FBS for the times specified. To assess DNA synthesis, HeLa cells were incubated with 12.5 μM bromo-deoxyuridine (BrdU) (BD Biosciences) immediately after phleomycin treatment, harvested 1-24 h later (as indicated), fixed in cold 80% ethanol, permeabilised in PBS/1% Tween-20, treated with 1.5 M HCl, and incubated with 5 μg.ml^{-1} of FITC-anti BrdU in 1% Tween-20/PBS, followed by simultaneous RNAse A treatment (0.5 mg.ml^{-1}) and propidium iodide (PI) staining (10 μg.ml^{-1} in 1% Tween-20/PBS). BrdU-labelled cells were detected by flow cytometry using a Becton Dickinson FACScalibur. In the mitotic entry assay, treated HeLa cells were allowed to recover in fresh medium containing 5% serum for the specified times before harvesting by trypsinisation, fixation in cold 80% ethanol and permeabilisation in 0.2% Triton-X/PBS. Cells were then incubated with anti-phospho-histone-3 antibody (1:200 in 1% BSA/PBS) followed by secondary antibody linked to Cy2 (1:200 in 1% BSA/PBS), and lastly simultaneously treated with RNAse A (0.5 mg ml-1) and stained with propidium iodide (PI) (10 μg.ml^{-1} in 1% BSA/PBS). Cells were analysed by flow cytometry using a Becton Dickinson FACScalibur and BD Cell QuestPro software, version 4.0.2.

Immunofluorescence and Confocal Microscopy. Transfected HeLa cells were seeded on chamber slides approximately 24 h before phleomycin exposure at a cell density of 2.4 x 10^4 cells.cm^{-2}. Cells were treated with 65 μM phleomycin for 1 h, fixed in 4% paraformaldehyde/PBS and permeabilised in 0.2% Triton X-100/PBS. Following blocking in 1% BSA/PBS, specimens were incubated with primary then secondary antibodies at a 1:200 dilution and mounted with 90% glycerol in PBS. Fluorescence images were captured using a Leica TCS SP2 spectral confocal microscope and analysed using Leica confocal software, version 2.5.

Colony Forming Assay. At 72 h post-transfection cells were treated with 13 μM phleomycin for 1 h. Following replacement of medium, colonies were allowed to grow for 10 days then fixed and stained with Diff Quik Stain kit (Lab Aids, Australia). Colonies were quantitated with Quantity One software (BioRad).

RESULTS AND DISCUSSION

Interaction between EDD and several components of DNA damage signalling prompted us to investigate the requirement for EDD in the G_1/S and intra-S phase checkpoints using siRNA-mediated EDD knockdown. Bromodeoxyuridine (BrdU) incorporation was used to measure DNA synthesis in HeLa cells transfected with either of two individual siRNAs targeted to different regions of EDD (Fig. 1). In the absence of DNA damage, 40% of EDD depleted cells were actively synthesising DNA compared to 29% of GFP siRNA control cells (Fig. 1B), suggesting a role for EDD in regulation of S phase entry and/or progression under normal growth conditions. Following phleomycin exposure a 45% reduction (from 29% to 16%) in the proportion of control cells synthesising DNA 1 h after treatment was observed. In contrast, there was only an 18% decrease (from 40% to 33%) in the S-phase fraction of EDD depleted cells (Fig. 1B), indicating a novel role for EDD in mediating the G_1/S and/or intra-S phase DNA damage checkpoints. This effect is accompanied by attenuated CHK2 Thr_{68} phosphorylation and kinase activity in EDD depleted cells[10] and mimics the effect of CHK2 depletion, which results in defective induction of G_1/S or intra-S phase checkpoints in response to double strand DNA breaks.[11]

To investigate if the compromised S phase DNA damage checkpoint in EDD depleted cells was mediated solely by attenuated CHK2 activity we measured DNA synthesis in cells depleted of EDD, CHK2 or EDD and CHK2 following DNA damage. CHK2 siRNA transfection of HeLa cells reduced CHK2 protein expression to approximately 10% of control levels (Fig. 2A). Simultaneous transfection of HeLa cells with both EDD and CHK2 siRNAs resulted in marked downregulation of both proteins although CHK2

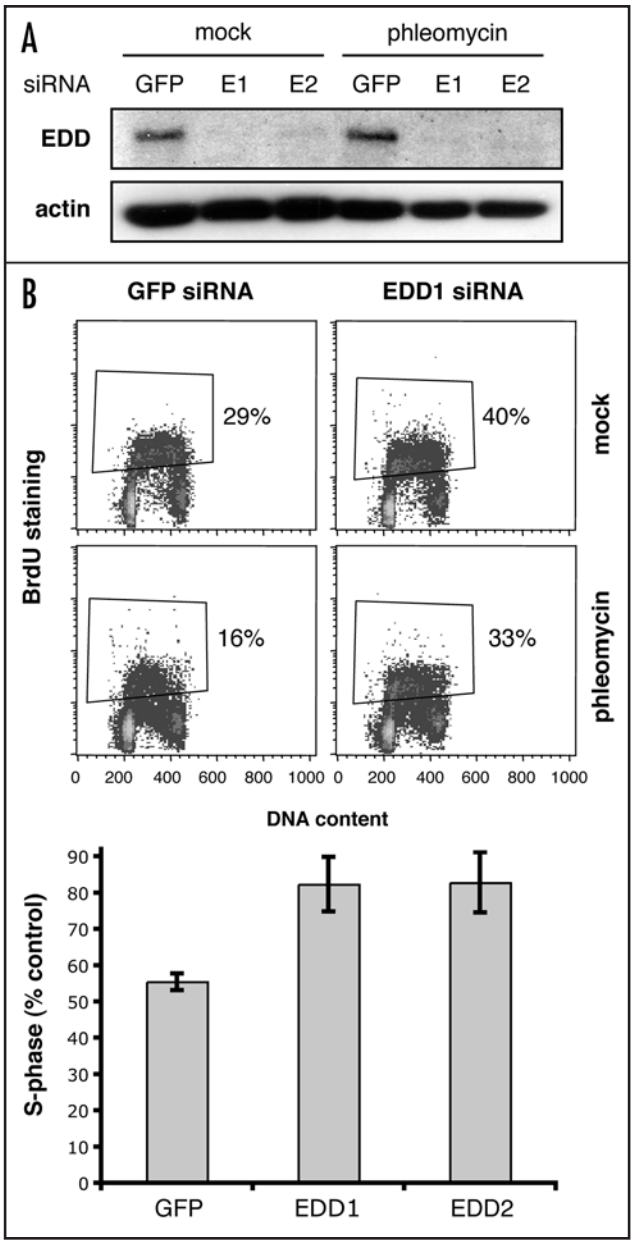

Figure 1. Effect of EDD depletion on DNA synthesis following phleomycin treatment in HeLa cells. Cells transfected with siRNA targeting EDD or GFP (control) and treated with phleomycin were labelled with BrdU for 1 h then stained with FITC-BrdU antibody and PI for flow cytometric analysis. (A) EDD expression in cells transfected with two different siRNA oligos targeting EDD (EDD1 or EDD2) or GFP (control). (B) Representative flow cytometry density plots showing BrdU incorporation vs DNA content (PI staining). Graph shows DNA synthesis expressed as percentage of BrdU-positive cells 1 h after phleomycin treatment, relative to untreated (mock) cells. Results represent the means of triplicate experiments ± SE (p=0.004, t-test).

knockdown was slightly less effective in dual siRNA transfections compared to individual transfections (Fig. 2A). Regardless, CHK2 Thr68 phosphorylation after phleomycin exposure was significantly lower in CHK2 siRNA transfected cells compared to those depleted of EDD alone, suggesting that CHK2 has been effectively inactivated. CHK2-depleted cells responded to DNA damage with a 35% decrease in S-phase fraction 24 h after phleomycin exposure, compared to a decrease of >55% in control cells (Fig. 2B,C), supporting a role for CHK2 in the G_1/S and/or intra-S phase DNA damage checkpoints. Further, the attenuated checkpoint response to phleomycin in cells depleted of CHK2 only was less severe than that observed in EDD-depleted cells (25% reduction in S-phase fraction) or cells depleted of EDD and CHK2 simultaneously (20% reduction in S-phase fraction) at this timepoint (Figs. 2B,C). Although the effect was less pronounced than in EDD-depleted cells, CHK2-depleted HeLa cells also displayed reduced long-term survival, as assessed by colony-forming assay after DNA damage (Fig. 2D). Together, these data indicate that at least some of the effects of EDD-depletion on the DNA damage response in HeLa cells may be mediated by CHK2-independent effects (see below).

Since other S phase checkpoint mediators e.g., ATM, BRCA1, CHK1 and CHK2, are also necessary for efficient G_2/M arrest after DNA damage[17,18] and we had previously observed increased mitotic entry in EDD-depleted cells 24 h after phleomycin treatment,[10] we hypothesised that EDD may also function in the G_2/M DNA damage checkpoint. We performed a detailed time-course analysis to investigate the role of EDD in the G_2/M checkpoint. A slight increase in the proportion of mitotic cells was seen 3 h after transfection with EDD siRNA (measured by phospho-Histone 3 expression) compared to the GFP siRNA control (e.g., 4.3% vs 3.3% of total cells - Fig. 3A), suggesting a role for EDD in regulation of mitotic entry under normal growth conditions. Efficient G_2/M checkpoint activation was observed in both control and EDD-depleted cells, with reduced mitotic entry observed within 3 h of phleomycin treatment (<10% of untreated levels) and maintained up to 18 h (Fig. 3B). However, EDD-depleted cells were not able to maintain this arrest and by 24 h post-phleomycin treatment a marked increase in the proportion of EDD-depleted cells entering mitosis was observed (Fig. 3B). This is reminiscent of the response of $Chk2^{-/-}$ thymocytes that efficiently activate the G_2/M checkpoint but fail to maintain arrest at later times.[18] By 48 h post-phleomycin exposure, EDD-depleted cells had resumed almost normal levels of mitotic entry (~75% of control), while control cells remained under partial G_2/M arrest (~36% of control) (Fig. 3B). These data indicate that EDD is necessary for maintenance of the G_2/M DNA damage checkpoint in HeLa cells, but is dispensable for initial activation of this checkpoint. Checkpoint adaptation, where cells reenter mitosis in the presence of DNA damage, has been suggested to promote genomic instability and tumourigenesis. A number of proteins have been implicated in G_2/M DNA damage checkpoint adaptation, including the yeast Chk2 homologue Rad53, Polo-like kinase 1 (Plk1) and the ATM and CHK2 substrate Che-1.[19-21]

Since we had previously reported preliminary evidence for a multi-nucleate phenotype in EDD depleted cells 24 h after phleomycin treatment[10] we next performed a detailed time-course analysis of this phenotype to further investigate the role of EDD in G_2/M checkpoint maintenance. Following phleomycin exposure there was a significant accumulation of EDD-depleted cells with >4N DNA content, increasing to 27% of the total cell population at 48 h compared with a ~10% increase in control cells (Figs. 4A,B). This >4N population comprised cells with a variety of nuclear aberrations including binucleation, irregular lobulated multiple nuclei, multi-micronucleation, and failed mitosis (Fig. 4D). Such nuclear morphology is often associated with mitotic catastrophe.[22] Further, we observed an approximate 2-fold increase in the sub-G_1 fraction of EDD-depleted cells at 18, 24 and 48 h post-phleomycin

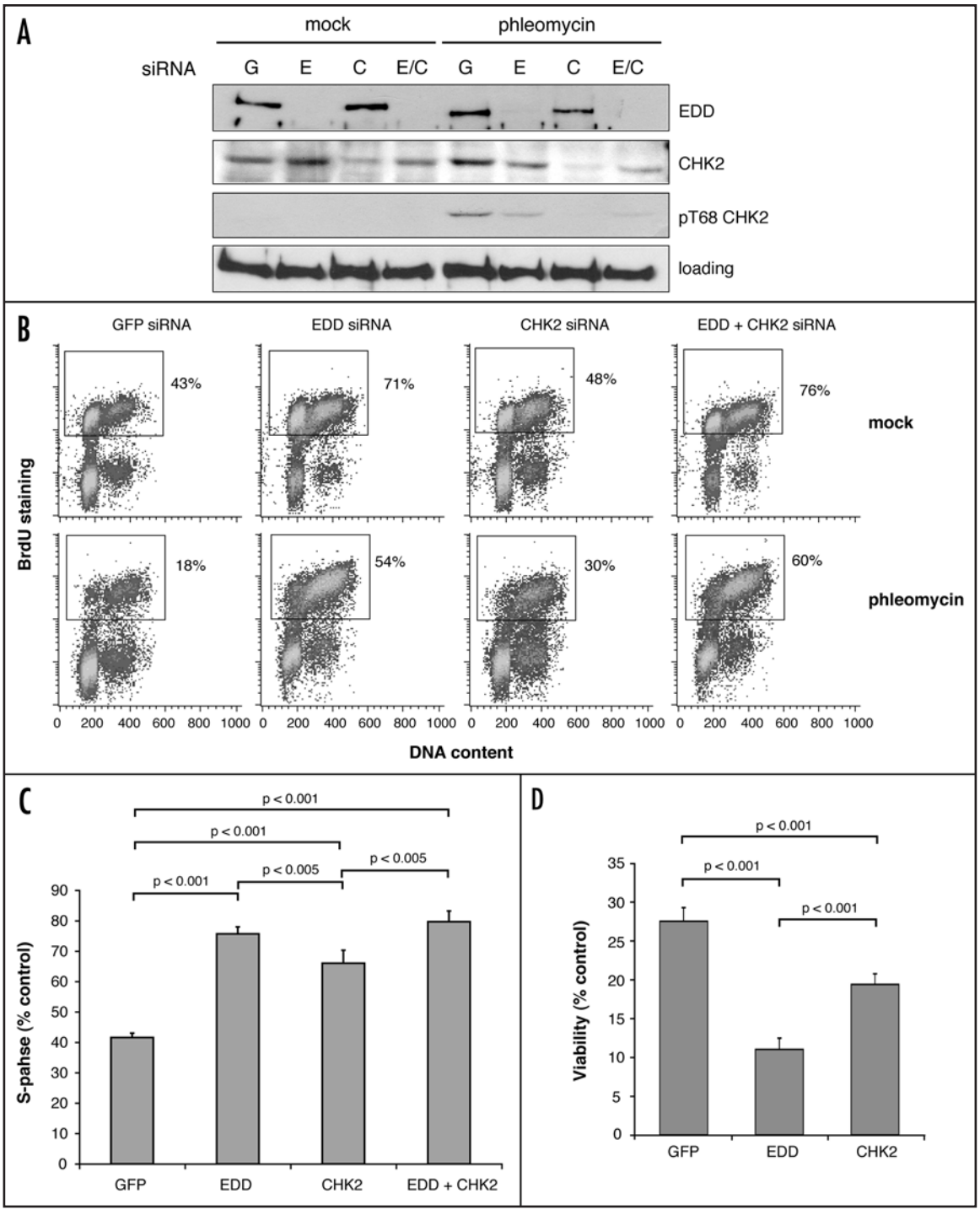

Figure 2. Effects of EDD or CHK2 depletion on DNA synthesis and long-term survival following phleomycin treatment in HeLa cells. (A) EDD and CHK2 expression in HeLa cells 72 h post transfection with siRNA targetted to GFP (G - control), EDD (E), CHK2 (C) or both EDD and CHK2 (E/C). (B) Representative flow cytometry density plots showing BrdU incorporation vs DNA content (PI staining). (C) Graph shows DNA synthesis expressed as percentage of BrdU-positive cells 24 h after phleomycin treatment, relative to untreated (mock) cells. Results represent the mean ± SE of triplicate experiments. (D) Long-term survival (measured by colony forming assay) in HeLa cells transfected with siRNA targeted to control (GFP), EDD, or CHK2. Following exposure to phleomycin, cells were allowed to form colonies for 10 days before quantitation. Viability is expressed relative to untreated (mock) cells and data represent the means ± SE (n=12).

exposure (Fig. 4C). This mirrored the accumulation of polyploid cells and suggested that a major component of cell death induced by DNA damage in these cells is due to an inability to complete mitosis. These data are consistent with decreased long-term viability of EDD-depleted cells following phleomycin treatment.[10]

CHK2 deficiency has also been associated with sensitisation to death by mitotic catastrophe and enhanced apoptosis in the absence of p53.[23] In p53-deficient cells, inactivation of the tetraploidy checkpoint allows cells to reenter the cell cycle with chromosomal aberrations that eventually result in mitotic catastrophe.[22,24] It has

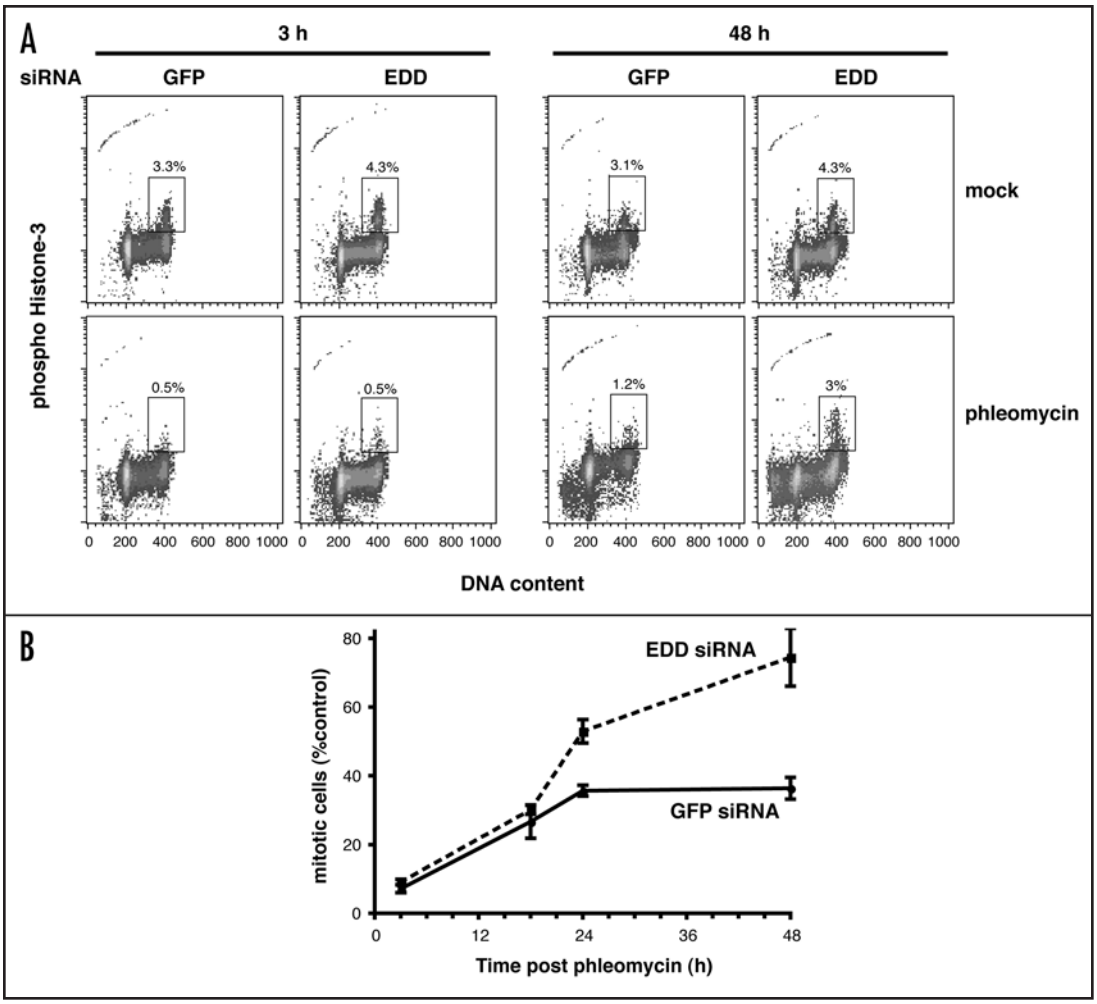

Figure 3. EDD is necessary for G_2/M checkpoint maintenance following phleomycin treatment in HeLa cells. Transfected cells treated ± phleomycin were left to recover for 3, 18, 24 or 48 h, stained with phospho-histone3 (p-H3) antibody and PI (DNA content) and analysed by flow cytometry. (A) Representative flow cytometry density plots from cells collected after recovering for 3 h or 48 h showing gates used to count mitotic (p-H3 positive) population. (B) Graph showing mitotic entry, expressed as percentage of P-H3 positive cells after phleomycin exposure relative to untreated (mock) cells. Results represent the means of five replicate experiments ± SE.

been suggested that mitotic catastrophe is the major death pathway in p53 mutant cells, preventing the survival of polyploid cells that could lead to aneuploidy and genomic instability.[23,24] The presence of an underlying population of control cells with aberrant DNA content in the absence of double strand DNA breaks (Fig. 4) is consistent with p53 impairment in HeLa cells,[25] and suggests that EDD depletion may act synergistically with p53 deficiency in the accumulation of polyploid cells in a DNA damage context.

A key regulatory point of DNA damage checkpoints is degradation of Cdc25 phosphatases following CHK2 and/or CHK1-mediated phosphorylation. Cdc25A controls S phase progression by activating cyclin A-E/Cdk2[26] and Cdc25C is an established mediator of the G_2/M DNA damage checkpoint via control of cyclin B/Cdk1 complex activity.[27] Consistent with increased DNA synthesis in EDD-depleted cells, we observed significantly higher expression of Cdc25A, irrespective of phleomycin treatment (Fig. 5A). Even though Cdc25A and Cdc25C expression were decreased in both EDD-depleted and control cells following phleomycin treatment, this response was attenuated in EDD-depleted cells. These effects are consistent with reduced CHK2 kinase activity (as evidenced by decreased Thr_{68} phosphorylation), compromised S-phase checkpoint, and the inability to maintain the G_2/M checkpoint in EDD-deficient cells (Figs. 1 and 5A). Attenuated CHK2 activation and increased Cdc25A/C levels may be sufficient to disturb the fine equilibrium between CDK inhibition and activation, tipping the balance toward the reestablishment of the positive feedback loop between CDK1 and Cdc25, eventually causing failure of G_2/M checkpoint maintenance in EDD-depleted cells.

Given the increased S-phase progression of EDD-depleted cells (Fig. 1), we assessed expression of the transcriptional activator E2F1, a key driver of S phase progression and mitotic entry. EDD depleted cells had higher E2F1 expression compared with control cells under both normal growth conditions and following phleomycin exposure (Fig. 5A). There were also markedly reduced levels of the Cdk2 inhibitors $p21^{WAF1/CIP1}$ (DNA damage-induced) and $p27^{Kip1}$ (integral cell cycle regulator) in EDD-depleted cells (Fig. 5A). Such perturbed expression of three critical G_1/S and intra-S phase checkpoint mediators indicate failure to effectively inactivate the cyclin/Cdk2 complex and hence DNA replication in EDD-depleted cells and likely contributes significantly to the dysregulated cell cycle progression and increased sensitivity to DNA damage observed in these cells. The identity of the E3 ligase(s) responsible for targeting E2F1 for ubiquitin-dependent degradation remains unknown but EDD is a potential candidate. Interestingly, CHK2 depleted cells did not exhibit the increased S-phase fraction observed in EDD-depleted cells in the absence of DNA damage (Fig. 2) and our preliminary data indicate that E2F1 expression is not altered in these cells (data not shown). Hence, the role of EDD in regulating S phase entry and/

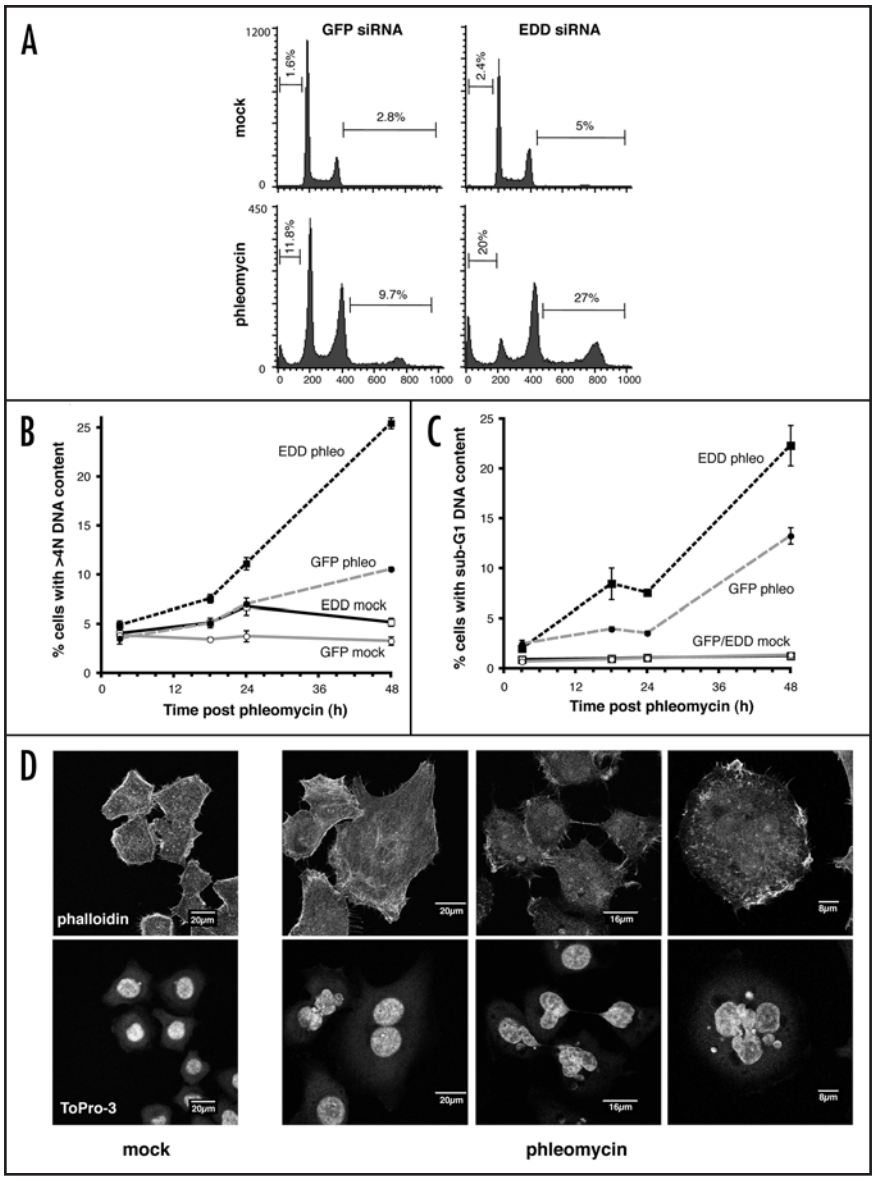

Figure 4. Polyploidy and mitotic catastrophe in EDD-depleted HeLa cells following phleomycin treatment. Transfected cells treated ± phleomycin were left to recover for 3, 18, 24 and 48 h. (A) Representative flow cytometry DNA histograms (using PI staining) 48 h after phleomycin exposure showing proportion of cells with sub-G_1 and >4N DNA content. (B) Accumulation of cells with aberrant (>4N) DNA content after phleomycin or mock exposure. (C) Accumulation of cells with sub-G_1 DNA content. Results shown in (B and C) represent the means of triplicate experiments ± SE. (D) Nuclear morphology of EDD-depleted cells 48 h after phleomycin or mock exposure. Cells were fixed and stained with TOPRO3 (DNA) and FITC-phalloidin (actin).

or progression under normal growth conditions may be mediated predominantly via CHK2-independent mechanisms, most likely via regulation of E2F1 expression by as yet unknown mechanisms.

Incorporating our data with published observations, we propose the following model for DNA damage checkpoint deficiency in EDD-depleted cells (Fig. 5B): decreased EDD expression leads to a combination of attenuated CHK2 activation, increased Cdc25A/C expression, increased E2F1 and decreased $p21^{WAF1/CIP1}$/$p27^{Kip1}$ expression, disrupting the normal regulatory network controlling cyclin/Cdk activity. As a result, EDD-depleted cells fail to attenuate DNA replication and reenter mitosis in the presence of damaged DNA. The significant decrease in cell survival after phleomycin exposure of EDD-depleted cells coincides with premature release from G_2/M arrest and the accumulation of a polyploid population. In the absence of an adequate p53 response in HeLa cells,[25] mitotic catastrophe and cell death eventually ensue. The phenotype of EDD-depleted cells is consistent with previous observations that radiosensitivity is not normally associated with defects in one checkpoint alone. Instead, simultaneous deficiencies in the G_1/S, intra-S and G_2/M checkpoints may act synergistically in the reduction of viability and in favouring genomic instability.[28]

We propose that EDD is an important mediator of the DNA damage response, potentially acting to regulate several key regulators of the signalling pathways controlling this response. Several recent gene expression profiling studies have reported an association between increased EDD expression and chemoresistance in a number of cancers (e.g., Refs. 29,30). Thus, the enhanced sensitivity to DNA damaging agents observed in EDD-depleted cells predicts that EDD

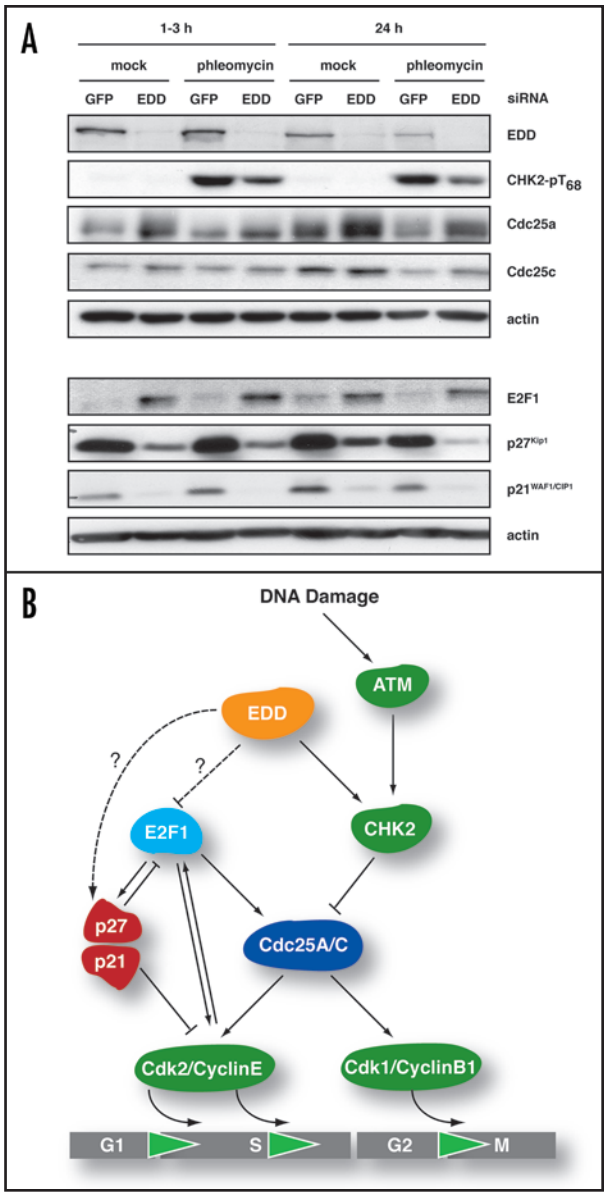

Figure 5. Disruption of cell cycle checkpoint regulatory network in EDD-depleted HeLa Cells. (A) Immunoblot analysis of expression of checkpoint mediators following 3 and 24 h recovery from phleomycin treatment, detected using the following antibodies: EDD polyclonal,[16] phospho-T68 CHK2 (Santa Cruz), Cdc25A (Neo-Markers), Cdc25C (Santa Cruz, H-6), E2F1 (Santa Cruz, C20), $p27^{Kip1}$ (BD Transduction Laboratories 610242), $p21^{WAF1/CIP1}$ (BD Transduction Laboratories 610234) and β-actin (Sigma, AC-15). (B) Schematic presentation of the proposed roles of EDD in the DNA damage checkpoint regulatory network.

expression levels could represent an important marker of tumour susceptibility to genotoxic agents and may represent a target for the development of novel therapeutic agents.

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