Inhibition of the Nedd8 system sensitizes cells to DNA Inter-strand crosslinking agents

Younghoon Kee1,*, Min Huang2, Sophia Chang1, Lisa A. Moreau2, Eunmi Park2, Peter G. Smith3, Alan D. D’Andrea2,*

1University of South Florida, Department of Cell Biology, Microbiology, and Molecular Biology, 4202 E. Fowler Avenue. Tampa, FL 33620
2Division of Genomic Stability and DNA Repair, Department of Radiation Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 450 Brookline Avenue, Boston, MA 02215
3Millennium Pharmaceuticals, Inc., 40 Landsdowne Street, Cambridge, MA 02139

*Corresponding Authors

Younghoon Kee
Mail: 4202 E. Fowler Avenue, BSF216 Tampa, FL 33620
Phone: 813-974-5352
Fax: 813-974-1614
Email: Ykee@usf.edu

Alan D. D’Andrea
Mail: 450 Brookline Avenue, Mayer 640, Boston, MA 02215
Phone: 617-632-2112
Fax: 617-632-5757
Email: Alan_Dandrea@dfci.harvard.edu

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ABSTRACT

The Fanconi Anemia (FA) pathway is required for repair of DNA interstrand crosslinks (ICLs). FA pathway-deficient cells are hypersensitive to DNA ICL-inducing drugs such as Cisplatin. Conversely, hyperactivation of the FA pathway is a mechanism that may underlie cellular resistance to DNA ICL agents. Modulating FANCD2 monoubiquitination, a key step in the FA pathway, may be an effective therapeutic approach to conferring cellular sensitivity to ICL agents. Here, we show that inhibition of the Nedd8 conjugation system increases cellular sensitivity to DNA ICL-inducing agents. Mechanistically, the Nedd8 inhibition, either by siRNA-mediated knockdown of Nedd8 conjugating enzymes or treatment with a Nedd8 activating enzyme inhibitor MLN4924, suppressed DNA damage-induced FANCD2 monoubiquitination and CHK1 phosphorylation. Our data indicate that inhibition of the FA pathway is largely responsible for the heightened cellular sensitivity to DNA ICLs upon Nedd8 inhibition. These results suggest that a combination of Nedd8 inhibition with ICL-inducing agents may be an effective strategy for sensitizing a subset of drug-resistant cancer cells.
INTRODUCTION

Cisplatin-based drugs have been used as a primary treatment for many types of cancers for more than 30 years. These drugs cause DNA damage, primarily via formation of interstrand DNA cross-linkages (ICL). ICLs are highly toxic to rapidly dividing cells, and cells that are unable to properly repair the damaged DNA die of apoptosis. However, the effectiveness of the therapy is often compromised largely because cancer cells develop resistance to the drugs (1). Elevated DNA repair pathways are observed in a subset of drug resistant tumor cells (2-3). Thus, understanding the cellular response mechanisms that regulate the activation of DNA repair pathways may provide a strategy for sensitizing some drug-resistant tumors. The DNA repair pathways that resolve DNA ICLs, such as Nucleotide Excision Repair (NER) and Homologous Recombination (HR), are coordinated by a DNA damage response pathway termed the Fanconi Anemia (FA) pathway (4).

Fanconi Anemia patients, who have a germline disruption of the FA pathway, exhibit congenital abnormalities, bone marrow failure, and genomic instability leading to cancers (4-5). Cells from FA patients display abnormally high sensitivity to DNA ICL-inducing agents, such as Cisplatin, Mitomycin C, and Melphalan. Fifteen FA genes have been identified to date (FANC-A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, and P). These act cooperatively in the FA pathway to coordinate the repair of DNA ICLs (6-8). The central regulatory event in the pathway is monoubiquitination of FANCD2, which requires S phase or DNA-damage induced activation of eight FA proteins (A, B, C, E, F, G, L, and M) that form a nuclear E3 ubiquitin ligase core complex. The activation of this FA core complex is preceded by a cascade of upstream DNA damage-induced signaling events involving the ATR and Chk1 kinases (4, 9). Monoubiquitinated FANCD2 is required for multiple steps during ICL repair, including the activation of the NER and TLS (Translesion Synthesis) steps (4), and the recruitment of HR repair factors such as BRCA1, BRCA2, RAD51, and FAN1(4).

Defects in the FA pathway also occur in somatic cells of non-FA individuals, causing diverse types of cancers (5, 10-12). Human tumors with FA gene mutations are particularly sensitive to ICL-inducing agents, such as Cisplatin and Mitomycin C (MMC). Conversely, restoration of a functional FA pathway is a mechanism for acquired cellular resistance to DNA ICL agents (10, 13-14). Interestingly, overexpression of FA genes accounts for drug-resistance in melphalan-resistant multiple myeloma (14-15). For these reasons, the FA pathway may be an effective target for chemosensitization in cancer treatment. Small molecule inhibitors of the FA pathway have been identified.
The Nedd8 system maintains the FA pathway by high-throughput platforms (13, 16), and an inhibitor of HSP90 has been shown to inhibit the FA pathway (17). Recently, the proteasome inhibitor Bortezomib, which is used for treating certain types of hematological tumors, was shown to inhibit the FA pathway, providing a mechanism for its anti-tumor effect (14, 18).

The ubiquitin-proteasome system regulates several essential cellular functions, including the cell cycle and DNA damage responses. Protein ubiquitination is achieved by a cascade of E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases, while reversal of ubiquitination is regulated by deubiquitinating enzymes. In addition to the ubiquitin system, eukaryotic cells utilize ‘ubiquitin-like modifiers’ or Ubls, such as SUMO, Nedd8, and ISG15, which provide additional layers of regulation for protein degradation. Nedd8 shares approximately 60% sequence identity with ubiquitin (19), and it is covalently attached to Lys residues on protein substrates in a manner similar to that of the ubiquitin system. The Nedd8 conjugation system consists of a single E1, a heterodimer of UBA3 and NAE1, two E2s, UBE2M (also known as UBC12) and UBE2F (20). The E3 for Nedd8 is not well characterized, and Nedd8 from the E2 can be directly transferred to Nedd8 substrates, including the cullin subunits of Cullin RING Ligase complexes (CRLs) (21). In humans, at least six cullin subunits (Cul1, 2, 3, 4A, 4B, 5) have been identified. These cullins form distinct CRL complexes with different substrate-specificity adaptors for protein degradation. Neddylation (protein modification by Nedd8) of the cullin subunits induces conformational changes within the CRL complexes, resulting in increased catalysis of substrate ubiquitination (20, 22). Therefore, the Nedd8 system affects a wide-range of cellular functions that are regulated by the CRL ligases. Recently, a pharmacological inhibitor of the Nedd8 system, MLN4924, was developed. MLN4924 has potent anti-tumor activity in cell culture as well as in a xenograft model (23), suggesting that the Nedd8 system may be an effective target for treating cancers.

To better understand the upstream signaling events that regulate FANCD2 monoubiquitination, we undertook a candidate-based siRNA screening that uses FANCD2 western blot as readouts. We found that knockdown of individual components in the Nedd8 conjugation system decreased DNA damage-induced FANCD2 monoubiquitination and foci formation, and CHK1 phosphorylation. The results are phenocopied by treatment of cells with MLN4924, the pharmacological inhibitor of NAE1. Consistent with these results, treatment with MLN4924, or siRNA-mediated depletion of the Nedd8 system, synergistically elevates cellular sensitivity to DNA damage.
ICL-inducing agents in various cell lines. These results suggest that a combination of a Nedd8 inhibitor with DNA ICL agents can be an effective therapeutic strategy against chemo-resistant cancer cells.

**Methods**

*Cell culture and chemicals*

HeLa, HCT116, 293T, U2OS, and MCF7 cell lines were cultured in DMEM media supplemented with 15% FBS and L-glutamine. These cell lines were obtained from ATCC and tested for microplasma contamination using Plasmocin (Invivo Gen). We also tested these cell lines for morphology using light microscopy, under low-density. Non-transformed LO2 liver cells and WI-38 lung human cell lines were grown in RPMI1640 media. 2008 cells (FA-F cells) corrected with FANCF cDNA was described previously (10). Cisplatin (SIGMA) was dissolved as 10mM stock solution in PBS and used as final concentration of 10µM. Hydroxylurea (HU; SIGMA) was dissolved as 2M stock solution in PBS and used as final concentration of 2mM. MMC (SIGMA) was dissolved in 70% Ethanol as 3.3mM stock concentration. MLN4924 was provided by Millenium Pharmaceuticals Inc., and dissolved in DMSO as a stock concentration of 10mM.

*siRNAs and antibodies*

siRNAs against UBA3 were as following: siRNA#1 (TGGCTCTGGTAGCCTGGGCATAGATG), siRNA#2 (CCGAGCACTGAATCTCTCCAGTTT), siRNAs against UBE2M were: siRNA#1 (GGGCTTCTACAAGAGTGGGAAGTTT), siRNA#2 (ACTCCATAATTTATGGCCTGCAGTA), siRNA against NAE1 was: AGCACAGTGGTATAGTGAACAAAT, siRNAs against UBE2F were: siRNA#1 (CGGAGGGTTTCTGTGAGAGACAAAT), siRNA#2 (ACTTCCGGAATAAAGTGGAGACAAAT), and siRNA against FANCM was: AAGCTCATAAAGCTCTCGGAA. Antibodies used in this study are as following: anti-FANCD2, anti-PCNA, anti-CDC25A antibodies (Santa Cruz Biotechnology), anti-phospho-CHK1(S317), anti-phospho-SMC1(S966), and anti-Claspin, anti-phospho-CHK2(T68) (Cell Signaling Technology), anti-CDT1, anti-53BP1 (Bethyl Laboratories), anti-RPA2 (EMD Biosciences), anti-γ-H2AX (Upstate), anti-γ-Tubulin (Sigma), anti-UBE2M, anti-BRCA1 (Abcam), and anti-phospho-NBS1(S343) (Oncogene) antibodies.
Immunofluorescence

HeLa cells were pretreated with extraction buffer (0.5% Triton X-100 in PBS buffer) on ice for 3 minutes prior to fixation with 4% paraformaldehyde. Anti-FANCD2, anti-53BP1, anti-RPA, and anti-γ-H2AX antibodies were used as primary antibodies. Alexa Fluor 488-conjugated and 594-conjugated antibodies (Invitrogen) were used as secondary antibodies. For the FANCD2 foci in Figure 3E, images were collected by a Zeiss Axiovert microscope equipped with a Yokogawa CSU-22 spinning disk confocal head and a 100x/1.45NA oil objective using Slidebook software (Intelligent Imaging Innovations, Denver, CO). 3D images were taken with 0.3 µm step size and then displayed as a maximal Z-projection.

Clonogenic and growth sensitivity assays

For clonogenic assays, HeLa cells were transfected with control, FANCM, or UBE2M siRNA in 6-well plates. 24 h after transfection, cells were re-plated in 6-well plates at 500 cells per well, followed by treatment with MMC at gradient concentration in the following day. HCT116 cells were treated with 20nM of MLN4924, and 24 hours later 20nM of MMC was added. Colonies were allowed to grow for 7–10 days, fixed with a solution containing 10% methanol and 10% acetic acid at room temperature for 15 min, and then stained with 1% crystal violet dissolved in methanol. Colonies of >50 cells were counted, and the surviving fraction was calculated and normalized to untreated control. The viable cells were determined by staining nucleic acids with a dye (CyQUANT; Molecular Probes, Eugene, OR) and subsequently analyzed by a fluorescence microplate reader (FL × 800; Bio-Tek Instruments, Winooski, VT) according to the manufacturer's protocol. For growth sensitivity assays using 2008 and 2008+FANCF cells, Cells growing in 96-well plate were treated with 20nM of MLN2949 for 24 hours and then exposed to Cisplatin for 4 more days. 20 µl of MTT (3- (4, 5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, SIGMA) at 5 mg/mL was added to each well and incubated at 37°C for 4 hours. The metabolic product was dissolved in a solution containing 10% SDS, 5% isopropanol and 0.01mol/L HCl and optical density at 565 nm was measured using an automatic microplate reader. The percentage of cell survival was determined by normalizing to solvent vehicle treated cells.

Cytogenetic analysis
Each cell line had one of its two plates treated with 20ng/ml MMC for 48 hours. Following treatment, the cells were exposed to colcemid (final concentration of 100ng/ml) for 2 hours, treated with a hypotonic solution (0.075M KCL) for 20 minutes and fixed with 3:1 methanol: acetic acid. Slides were stained with Wright’s stain and when possible, 50 metaphase spreads were scored for aberrations. Metaphase spreads were observed using a Zeiss Axio Imager microscope and captured using CytoVision software from Applied Imaging.

RESULTS

Depletion of the Nedd8 conjugation system abrogates FANCD2 monoubiquitination

To identify upstream factors that affect the damage-inducible FANCD2 monoubiquitination (FANCD2-ub), we undertook a candidate-based siRNA screen. Various DNA damaging agents (UV, HU, Cisplatin, IR) induce FANCD2 monoubiquitination, and there are few known upstream factors that regulate DNA damage-induced FANCD2 monoubiquitination including the FA core complex members, ATR, and CDK1(9, 24). We hypothesized that there might be other ubiquitin-mediated signaling events upstream of the monoubiquitination of FANCD2. Depletion of UBA3, an E1 component of the Nedd8 conjugation system, strongly inhibited damage-inducible FANCD2 monoubiquitination (FIGURE 1A, B). To further test if other components in the Nedd8 conjugation system also affect FANCD2 monoubiquitination, we tested knockdown of UBE2M, an E2 conjugating enzyme for Nedd8. UBE2M depletion similarly inhibited FANCD2 monoubiquitination after UV (FIGURE 1D and 1F; HCT116 and HeLa cells, respectively), or after psoralen/UVA treatment (FIGURE 1E and 1G; HCT116 and HeLa cells, respectively). The level of CHK1 phosphorylation (p-CHK1) was also consistently lower when the Nedd8 conjugation pathway was inhibited. The level of monoubiquitinated PCNA (PCNA-ub) remained similar upon depletion of UBE2M, suggesting that the effect on FANCD2 monoubiquitination is specific. Phosphorylation of other DNA damage response proteins such as H2AX, BRCA1, and SMC1 were elevated or remained at a similar level upon UBE2M knockdown, suggesting that knockdown of UBE2M caused some level of DNA damage and did not disrupt the overall DNA damage response. Knockdown of UBA3 or UBE2M did not significantly affect cellular proliferation, as measured by BrdU staining (FIGURE 1C). Consistent with a previous report (20), knockdown of UBE2M modestly increased the G2/M peak in the cell cycle distribution analysis (Supplementary FIGURE 1).
The monoubiquitinated form of FANCD2 localizes to chromatin and forms damage-inducible foci. Consistent with the reduction of FANCD2 monoubiquitination, foci formation of FANCD2 was also reduced upon UBE2M knockdown (FIGURE 2). Interestingly, foci formation of other DNA damage response proteins, such as γ-H2AX, 53BP1, and RPA, were not affected, suggesting that the effect is specific for FANCD2. Since other DNA damage response proteins are not affected, the effect on FANCD2 is not simply due to a disruption in the cell cycle. Knockdown of NAE1, the catalytic subunit of the Nedd8 E1 activating enzyme also inhibited damage-inducible FANCD2 monoubiquitination and CHK1 phosphorylation, whereas knockdown of UBE2F, a Nedd8 E2 that participates in neddylation of a smaller subset of cullins (20), did not affect the level of FANCD2 monoubiquitination (Supplementary FIGURE 2). Depletion of USP1, a deubiquitinating enzyme targeting FANCD2, elevates the level of FANCD2 monoubiquitination (25). Surprisingly, depletion of UBE2M did not reduce the FANCD2 monoubiquitination that is up-regulated by USP1 depletion (Supplementary FIGURE 3), suggesting that the Nedd8 inhibition specifically abrogates FANCD2 monoubiquitination that is induced by exogenous DNA damage. Altogether, these results suggest that the Nedd8 conjugation system is required for DNA damage-inducible FANCD2 monoubiquitination and CHK1 phosphorylation.

**Nedd8 inhibitor MLN4924 inhibits DNA damage-induced activation of the FA pathway**

Recently, a pharmacological inhibitor of the Nedd8 system was described (23). This investigational agent, MLN4924, targets the NAE1 component of the Nedd8 E1 enzyme, stabilizes substrates of CRL E3 ligases such as CDT1, induces DNA re-replication and checkpoint activation, and triggers apoptosis (23, 26-27). We tested whether MLN4924 phenocopies the siRNA results described above. We pre-treated cells with MLN4924 and then exposed them to UV or psoralen+UVA (PUVA) for variable times (FIGURE 3A and 3B; HCT116 and HeLa cells, respectively, and Supplementary FIGURE 4 for HeLa treated with UV). Consistent with previous reports, phosphorylation of BRCA1, SMC1, CHK1, CHK2, γ-H2AX, and monoubiquitination of FANCD2 and PCNA were induced by the compound treatment (compare 0 time points between DMSO and MLN4924). When combined with DNA damaging agents, the compound specifically inhibited the sustained activation of p-CHK1 and FANCD2-ub, whereas activation of other DNA repair proteins remained unaffected. These effects were observed in other cell types, such as U2OS (Supplementary FIGURE 5), MCF7 (Supplementary FIGURE 6), and FA-deficient ovarian tumor
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Line 2008 (FIGURE 4C right panel). Longer treatment of MLN4924 was required to observe the reduced level of p-CHK1 and FANCD2-ub at the lower inhibitor dosage (Supplementary FIGURE 7). In a reverse experiment, when MLN4924 was added after exogenous DNA damage, p-CHK1 and FANCD2-ub levels were suppressed, while p-CHK2, PCNA-ub, and g-H2AX remained unaffected (FIGURE 3C). The effect of MLN4924 was dose-dependent, as shown in FIGURE 3D. One discrepancy between the siRNA and MLN4924 results is that the spontaneous activation of DNA checkpoints was not observed following Nedd8 knockdown by siRNA. Perhaps longer treatment of cells with the siRNAs (>60 hours) may elicit secondary effects that suppress activation of DNA checkpoint completely.

Consistent with the siRNA results, MLN4924 effectively suppressed DNA damage-induced foci formation of FANCD2, whereas foci formation of g-H2AX and 53BP1 was not affected (FIGURE 3E). Treatment with MLN4924 without additional damage caused foci formation of these DNA damage response proteins, to varying degrees, suggesting that the drug itself triggers DNA damage. Altogether, MLN4924 phenocopied the siRNA results in which the inhibition of the Nedd8 conjugation system suppresses DNA damage-induced FANCD2 monoubiquitination and CHK1 phosphorylation. Therefore, Nedd8-targeting inhibitors such as MLN4924 may sensitize cancer cells to DNA damaging agents, particularly to agents that generate DNA ICLs, which require the functional FA pathway for repair.

We previously reported that the FANCM subunit of the FA core complex is ubiquitinated and degraded during mitosis, mediated by the CRL$^β$-TRCP E3 ubiquitin ligase. FANCM degradation appear to be one mechanism for cell-cycle specific activation of the FA pathway (28). The failure of the mitosis-specific degradation of FANCM leads to improper chromatin loading of the FA core complex and heightened cellular sensitivity to MMC. Since the degradation is mediated by a CRL ligase, we reasoned that inhibition of Nedd8 conjugation may disrupt the timed degradation of FANCM during mitosis. FANCM was phosphorylated and degraded when cells were released into the mitotic phase of the cell cycle (FIGURE 3F, lanes 1-4), and treatment (3-4 hours) of proteasome inhibitor MG132 inhibited the degradation of FANCM (Lanes 5-8). Treatment with MLN4924 similarly stabilized FANCM (lanes 9-12), suggesting that the CRL ligase activity is inhibited by the Nedd8 inhibitor. From these results, we suggest that the perturbed degradation of FANCM during mitosis by inhibition of Nedd8 is one mechanism reflected by heightened cellular sensitivity to the Nedd8 inhibition.
Inhibition of the Nedd8 conjugation system hypersensitizes cancer cells to DNA ICL agents

We next tested whether inhibition of the Nedd8 system causes hyper-sensitization of cells to DNA ICL agents. Knockdown of UBE2M in HeLa cells significantly reduced cell growth after Mitomycin C (MMC) treatment, as shown by clonogenic assay (FIGURE 4A). Knockdown of FANCM, a FA gene shown to be required for resistance to MMC (29), was tested as a positive control. Treatment of HCT116 colon cancer cell lines with MLN4924 significantly reduced cell survival in combination with the Cisplatin (FIGURE 4B; two independent experiments). These results demonstrate that the Nedd8 system is required for cellular resistance to the DNA crosslinking agents. The degree of sensitization appears to be preferential to the transformed cell lines, as two normal human diploid cells, LO2 and WI-38 cells were not significantly sensitized to Cisplatin by the Nedd8 inhibition (Supplementary Figure 8). The MLN4924-mediated sensitization of cells to DNA ICL agents resulted, at least partially, from inhibition of the FA pathway, since FANCF-proficient and ICL-resistant ovarian tumor cells (2008+F) showed enhanced sensitivity to MLN4924, compared to FANCF-deficient and ICL-sensitive counterpart 2008 cells (FIGURE 4C). The slight sensitization of the FA-deficient tumor cells 2008 by MLN4924 to Cisplatin suggests that MLN4924 disrupt targets other than the FA pathway, including the proteins of the ATR-CHK1 signaling pathway. To directly demonstrate the reduced DNA repair capacity upon Nedd8 inhibition we performed cytogenetic analysis of 293T cells. We observed synergistic effects of depleting UBE2M (FIGURE 4D), or treating MLN4924 (FIGURE 4E), with MMC treatment in inducing chromosomal aberrations, suggesting that Nedd8 inhibition compromised cellular DNA repair capacity upon DNA ICLs. Altogether, these results demonstrate that inhibition of Nedd8 conjugation system is disrupts cellular resistance to DNA ICLs.

DISCUSSION

We have demonstrated that inhibition of the Nedd8 conjugation system sensitizes various cancer cells to DNA ICL-inducing agents. Mechanistically, Nedd8 inhibition abrogated DNA damage-inducible activation of CHK1 phosphorylation and FANCD2 monoubiquitination. Previous reports have shown that treatment with MLN4924 activates DNA damage responses, such as CHK1 phosphorylation (23, 26). While our study is consistent with this result, it further demonstrates that
damage-induced activation of CHK1 and FANCD2 is suppressed upon Nedd8 inhibition. Thus, the Nedd8 conjugation system is required for sustained activation of CHK1 and FANCD2.

The suppression of CHK1 phosphorylation and FANCD2 monoubiquitination was not due to alteration in cell cycle progression. Other DNA damage responses that occur during S/G2 phases, such as PCNA monoubiquitination, remain unchanged upon Nedd8 inhibition. Furthermore, phosphorylation of ATM substrates, such as CHK2, 53BP1, NBS1, BRCA1, and γ-H2AX, remained unaffected or even elevated under the same conditions. We therefore conclude that a Nedd8-mediated protein modification event is required for damage-inducible ATR/CHK1 activation and for subsequent FANCD2 monoubiquitination (FIGURE 5).

How Nedd8 inhibition suppresses CHK1 phosphorylation and FANCD2 activation requires further investigation. Increasing evidence supports a strong interaction between ATR/CHK1 signaling and the FA pathway. ATR and CHK1 function is required for induction of FANCD2 monoubiquitination (9, 30), and CHK1-mediated phosphorylation of FANCE is required for cellular resistance to MMC (31). Consistent with reports that CHK1 may function upstream of FANCD2, siRNA-mediated depletion of CHK1 partially suppressed damage-inducible FANCD2 monoubiquitination (data not shown). The observation that CHK1 phosphorylation is still suppressed by Nedd8 inhibition, regardless of the status in FANCD2 monoubiquitination in FANCF-deficient 2008 cell lines (FIGURE 4C), further supports the hypothesis. These data altogether suggest that the abrogation of FANCD2 monoubiquitination upon Nedd8 inhibition may be due, at least in part, to the failure in CHK1 activation.

The primary role of Nedd8 is to activate CRL ligases (21). Accumulation of CDT1, a CRL substrate and a licensing factor for the pre-replication (pre-RC) complex, is a key cytotoxic effect of MLN4924 (23, 26-27). Accumulation of CDT1 induces DNA re-replication and triggers DNA damage and CHK1 activation (32-33). CDT1 degradation by the CRL ligase ensures that DNA replication origins fire only once per cell cycle (34-35). Even though Nedd8 inhibition caused an accumulation of CDT1, it failed to activate CHK1 (FIGURE 3). This result suggests that damage-inducible CHK1 activation requires additional events that are mediated by the Nedd8 conjugation system.

Besides the cullin subunits of the CRL complexes, p53 is also modified by neddylation, resulting in inhibition of its activity (36). The effect of Nedd8 inhibition in suppressing the DNA damage response is unlikely to be mediated via p53 modification, since our results were observed in
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several cell types regardless of p53 status (FIGURE 3 and supplementary FIGURE 5 and 6). We previously reported that a CRL ligase containing β-TRCP mediates mitotic degradation of FANCN (28). The perturbation of timed degradation leads to disruption of the FA core complex recruitment cycle and increase in the cellular sensitization to MMC. Based on our evidence that MLN4924 disrupts the mitotic degradation of FANCN (Figure 3F), we propose that Nedd8 inhibition disrupts FA pathway, at least in part, through perturbation of mitotic FANCN degradation. However, we cannot rule out the possibility that there may be additional CRL ligases and substrates that regulate the FA pathway.

Since various cellular signaling pathways affect cellular sensitivity to DNA ICL agents, a pharmacologic inhibitor of such pathways may improve current chemotherapeutic regimen. Previous reports have shown that proteasome inhibitors sensitize cells to DNA damaging agents, suggesting that these agents may be synergistic in combination therapy (37-38). Proteasome function is required for activation of DNA damage responses including the FA pathway (14, 18), providing a basis for the proteasome-mediated sensitization of cells to DNA damaging agents. The Nedd8-CRL ligases account for approximately 20% of proteasome-mediated protein degradation in cells (23). Furthermore, inhibition of proteasome, but not of the Nedd8 conjugation, causes over-accumulation of overall ubiquitin conjugates in cells (not shown), suggesting more selectivity of the Nedd8 inhibition. Thus, inhibition of the Nedd8 system, in combination with DNA damaging agents, and more specifically ICL agents, might offer an alternative strategy which could provide more specificity in targeting the DNA damage response. Furthermore, our work suggests that the proteasome-mediated suppression of the FA pathway could be largely due to the suppression of the Nedd8 and CRL-mediated protein degradation. Further investigation of the mechanism involved in this pathway will provide better insight into DNA damage signaling, and may provide a new avenue for the design of more specific anti-cancer drugs.
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FIGURE LEGENDS

Figure 1. Knockdown of Nedd8 conjugation system disrupts FANCD2 monoubiquitination and CHK1 phosphorylation. A. Representative western blot for the candidate siRNA screens. HeLa cells were treated with siRNAs against various E3 ligase components. Forty eight to sixty hours later cells were treated with UV (30J/m²), then the cells were harvested and lysed for the western blot analysis. B. HeLa cells were treated with UBA3 siRNA#1, followed by UV treatment. Cells were harvested at indicated time points. C. HeLa cells were transfected with the siRNAs and 48 hours later cells were incubated with 10µM BrdU for 40 minutes. Integrated BrdU was visualized using FITC-conjugated BrdU antibody and quantified by FACS analysis. D. HCT116 cells were treated with two different UBE2M siRNAs, followed by UV (30J/m²) treatment before harvested for western blot analysis. E. HCT116 cells were treated with UBE2M#1 siRNA, followed by PUVA (Psoralen+UVA) treatment, then harvested at indicated time points for western blot analysis. F and G. HeLa cells were treated with UBE2M siRNAs followed by UV (30J/m²) and PUVA treatment, respectively, for western blot analysis.

Figure 2. Knockdown of UBE2M abrogates damage-inducible FANCD2 foci formation. HeLa cells were treated with control or UBE2M#1 siRNA for 48 hours, followed by HU (2mM) treatment for 12 hours for analyzing damage-inducible foci formation.

Figure 3. MLN4924 suppresses damage-inducible CHK1 phosphorylation and FANCD2 monoubiquitination. A. HeLa cells were treated with 1µM of MLN4924 or DMSO vehicle for 20 hours, followed by Psoralen+UVA (PUVA) treatment, and cells were harvested at indicated time points for western blottings. B. HCT116 cells were treated with 3µM of MLN4924 or DMSO vehicle for 18 hours, followed by UV (30J/m²) treatment, and cells were harvested at indicated time points for western blottings. C. HeLa cells were treated with Cisplatin (10µM) for 16 hours,
followed by treatment with MLN4924 (2µM) or DMSO for indicated times before harvested for western blottings. **D.** HeLa cells were treated with indicated concentrations of MLN4924 for 12 hours followed by 12 hour-treatment of 10µM Cisplatin. **E.** HeLa cells were treated with MLN4924 (1µM) or DMSO for 16 hours, followed by HU (2mM) treatment for 20 hours before fixation for the immunofluorescence assay. Experiments were performed in triplicate and the average numbers of foci were plotted as graphs. **F.** HeLa cells were synchronized in S phase using double thymidine block, then the cells were released into fresh medium containing nocodazole. During the last 3 hours of incubation cells were treated with DMSO, MG132, or MLN4924 before harvested. The lysates were analyzed by western blottings. Protein band intensities were measured and plotted as graphs.

**Figure 4. Inhibition of the Nedd8 conjugation system sensitizes cells to DNA crosslinking agents and PARP1 inhibitor.** **A.** HeLa cells were treated with siRNAs against control, FANCM, or UBE2M for 48 hours, followed by MMC treatment, and the cells were incubated for 7-10 days before fixation (see Methods for detail). **B.** HCT116 cells were treated with 20nM of MLN4924 or DMSO control, and next day MMC was added for further incubation of 7 days before fixation. **C.** Left panel. 2008 ovarian cancer cells harboring vector (2008) or FANCF cDNA (2008+F) were treated with 20nM of MLN4924, followed by treatment with indicated amount of Cisplatin. After 5 days, viability of cells was measured as described in Methods. Right panel. The 2008 and 2008+FANCF cells were treated with 2µM of MLN4924 for 20 hours, followed by UV (30J/m²) irradiation, and the cells were harvested at indicated time points. **D and E.** Chromosomal aberrations were measured in 293T cells that were treated with 100nM of MLN4924 (D) or transfected with siRNAs (E), followed by MMC treatment for 3 days.

**Figure 5. A protein neddylation event is required upstream of the FA pathway.** A schematic model for the Nedd8-mediated activation of the DNA damage-inducible FANCD2 monoubiquitination. While interruption of the Nedd8 conjugation system suppresses the ATR-mediated signaling pathways, particularly CHK1 phosphorylation and FANCD2 monoubiquitination, ATM-mediated pathways or PCNA ubiquitination remain unaffected. We hypothesize that specific protein neddylation events, most likely the activation of specific CRL E3 ligases, is required for damage-inducible CHK1 phosphorylation and FANCD2 monoubiquitination. We suggest that
The Nedd8 system maintains the FA pathway disruption in the CRL-mediated destruction of FANCM is one mechanism leading to the abrogation of the FA pathway.
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Kee et al, FIGURE 2
Inhibition of the Nedd8 system sensitizes cells to DNA interstrand crosslinking agents

Younghoon Kee, Min Huang, Sophia Chang, et al.

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