

Inhibition of NEDD8-Activating Enzyme Induces Rereplication and Apoptosis in Human Tumor Cells Consistent with Deregulating CDT1 Turnover

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Abstract

Loss of NEDD8-activating enzyme (NAE) function by siRNA knockdown or inhibition by the small molecule NAE inhibitor MLN4924 leads to increased steady-state levels of direct Cullin-RING ligase (CRL) substrates by preventing their ubiquitination and proteasome-dependent degradation. Many of these CRL substrates are involved in cell cycle progression, including a critical DNA replication licensing factor CDT1. Cell cycle analysis of asynchronous and synchronous cultures after NAE inhibition revealed effects on cell cycle distribution and activation of DNA break repair signaling pathways similar to that reported for CDT1 overexpression. The siRNA knockdown of cullins critical for the turnover of CDT1 recapitulated the aberrant rereplication phenotype while CDT1 knockdown was suppressing. Although NAE inhibition leads to deregulation of many CRL substrates, these data demonstrate that CDT1 accumulation mediates the DNA rereplication phenotype resulting from loss of NAE function. DNA rereplication is an unrecoverable cellular insult and the small molecule inhibitor MLN4924, currently in phase I trials, represents an unprecedented opportunity to explore this mechanism of cytotoxicity for the treatment of cancer. *Cancer Res*; 71(8); 3042–51. ©2011 AACR.

Introduction

Covalent attachment of ubiquitin to cellular proteins regulates many diverse cellular processes (1, 2). The effects of tagging proteins with other ubiquitin-like proteins (Ubl) such as NEDD8 and SUMO have also been described (3, 4) and include modulation of protein activity, intermolecular interactions, and subcellular localization (5). Typically, Ubls are conjugated to their substrates via 3-step enzymatic cascades exemplified by the well-described ubiquitination cascade (6). NEDD8 is activated by the E1 NEDD8-activating enzyme (NAE), and transferred to 1 of 2 specific E2s before conjugation to its substrates (7, 8). The best characterized substrates modified by NEDD8 are the cullin family of proteins (CUL-1, 2, 3, 4A, 4B, 5, and 7) that are components of a family of multisubunit E3 ligases known as cullin-RING ligases (CRL; refs. 9, 10). The CRL family mediates substrate ubiquitination by recruiting a targeted protein via substrate recognition modules at the cullin N-terminus and the ubiquitin-charged E2 at the cullin C-terminus (11). NEDD8 modification of cullin

proteins is essential for activation of CRL activity by mechanisms that are still being investigated (12–14). Many substrates of CRLs play important roles in cell growth and survival, demonstrating the importance of the NEDD8 pathway in cancer (12, 13).

Recently, we showed that MLN4924, a potent and selective small molecule inhibitor of NAE, suppresses the growth of human tumor cell lines and xenografts in nude mice (15). At the cellular level, NAE inhibition disrupted the turnover of known CRL substrates, and defects in DNA synthesis regulation were the predominant phenotype. Eukaryotic cells maintain their genomic stability by ensuring that DNA replication occurs only once during a cell cycle through tight control of the prereplication complex (pre-RC; refs. 16–18). Following initiation of DNA synthesis, failure to disassemble the pre-RC can result in relicensing and refiring of previously fired origins of replication, a deleterious process referred to as DNA rereplication (19–22). There are redundant mechanisms to prevent rereplication, such as degrading ORC1 and CDT1 which are components of the pre-RC and also CRL substrates. Modulating CDT1 levels by overexpression or depletion of geminin, the endogenous inhibitor of CDT1, has been shown to lead to rereplication, activation of DNA damage repair pathways, genomic instability and apoptosis (22, 23).

In this study we show that, despite blocking the turnover of a broad array of important cell cycle regulators, the dominant phenotype of NAE inhibition is an S-phase arrest and the appearance of rereplicated DNA. We attribute the induction of DNA rereplication to the inability of cells to properly degrade components of the pre-RC complex, specifically CDT1. In addition, we show that the DNA damage response resulting

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from NAE inhibition-dependent DNA rereplication is consistent with the sequential induction of both single- and double-strand DNA breaks through the ATM/ATR kinase pathways (24). Ultimately, the inability to cope with rereplication and failure to repair the damaged DNA leads to apoptosis.

Materials and Methods

Cell culture and Western blot analysis

HCT-116 cells and MCF10A cells were used as previously described (15). Immunoblotting assays were performed as previously described (15) with primary antibodies as follows: CDT1, NEDD8, Emi1, NRF2, Cullin1, and Cullin4a/b (Millennium); pH3 (Ser 10), pCHK1 (S317), pRAD17 (S645), pCHK2 (T68), pNBS1 (S343), cleaved PARP, and cleaved caspase-3 (Cell Signaling); p27 and Cyclin E (BD Biosciences); ORC1, CDC6 and Geminin (Santa Cruz); p21 and tubulin (Lab Vision/NeoMarkers); pH2AX (Ser 139; Millipore); Cyclin D1 (Stressgen); and NRF2 (Epitomics). Secondary antibodies to rabbit IgG or mouse IgG (Santa Cruz) were used as appropriate.

Cell cycle analysis and synchronization

Cell cycle analysis was performed as previously described (15). For synchronization experiments, cells were treated with 5 $\mu\text{mol/L}$ aphidicolin (Sigma) for 16 hours at 37°C prior to release into either dimethyl sulfoxide (DMSO) or concentrations of MLN4924 as indicated.

Bromodeoxyuridine incorporation analysis

Cells were pulsed with 10 $\mu\text{mol/L}$ bromodeoxyuridine (BrdUrd; BD Biosciences) for the final 30 minutes of the indicated MLN4924 drug treatment time and processed according to manufacturer's protocol (BD Biosciences). Labeled cells were analyzed for PI and BrdUrd staining on a Becton Dickinson FACS Calibur flow cytometer using Winlist software (Verity).

RNAi

Transfections were performed with 50 nmol/L siRNA oligonucleotide duplexes on HCT-116 cells using Lipofectimine 2000 (Invitrogen) according to the manufacturer's instructions, with On-TARGETplus SMARTpools: GL2 (D-001100-01), CDT1 (L-003248-00), CUL1 (M-004086-01), CUL4A (M-012610-00), and CUL4B (M-017965-00; Dharmacon RNAi Technologies) and harvested 48 hours after transfection.

Results

Inhibition of NAE stabilizes CRL proteins responsible for cell cycle progression

The small molecule NAE inhibitor MLN4924 disrupts the ubiquitination and subsequent degradation of many CRL target proteins (15). Many CRL substrates are important for proper cell cycle progression, including p27 (25, 26), p21 (27), Emi1 (28), and cyclins (29, 30). Modulating any of these substrates by RNAi or overexpression can yield varied effects on cell cycle distribution. HCT-116 colon carcinoma cells were treated with 1 $\mu\text{mol/L}$ MLN4924, resulting in rapid inhibition

of NEDD8-cullin conjugation and diminished CRL activity, which coincided with a reciprocal increase in cellular protein levels of various cell cycle regulators (Fig. 1A). We therefore were interested in investigating which of these substrates may be impacting the dominant cell cycle phenotype following NAE inhibition.

Flow cytometric analysis of HCT-116 cells treated for 24 hours with even as low as 0.04 $\mu\text{mol/L}$ MLN4924 revealed, a growing subpopulation in S-phase as well as a smaller subset having greater than tetraploid (>4N) DNA content (Fig. 1B). Increasing MLN4924 concentrations exacerbated this cell cycle phenotype with an increase in cells with more than 4N DNA. At higher concentrations the accumulation of cells having more than 4N DNA content is diminished and is likely the result of increased cell death consistent with the appearance of cleaved caspase-3 and cleaved PARP as previously reported (15), as well as an increase in Annexin V positive cells following MLN4924 treatment (Supplementary Fig. S1). A BrdUrd-incorporation time-course experiment was conducted on asynchronous HCT-116 cells treated with MLN4924 to measure the amount of DNA synthesis in the accumulating S-phase cells following treatment. Greater than 90% of the cells treated with MLN4924 incorporated BrdUrd as early as 8 hours, compared to only 59% of cells treated with DMSO. The increased BrdUrd incorporation observed with the MLN4924-treated cells was sustained out to 24 hours, suggesting persistent DNA synthesis occurred within these cells, leading to increased DNA content (see Fig. S2 in the Supplementary Material). Cells accumulating in S-phase, failing to enter mitosis, and collecting DNA with more than 4N content are consistent with loss of NAE function causing DNA rereplication (15, 22).

The pre-RC component CDT1 is a CRL1^{Skp2} and CRL4-DDB1^{Cdt2} substrate and has been shown to induce DNA rereplication in cells when overexpressed (22, 23, 31, 32). We observed a dose-dependent increase in CDT1 levels following MLN4924 treatment (Fig. 1C) and the increased steady-state levels of CDT1 protein correlated with the increase in cells with more than 4N DNA content (Fig. 1B and C). We analyzed additional components within the pre-RC (ORC1 and CDC6) following MLN4924 treatment (33, 34) and found their protein levels only modestly affected (Fig. 1C).

NAE inhibition deregulates CDT1 in S-phase leading to increased DNA synthesis and rereplication

To further examine the effects of NAE inhibition on cell cycle progression we synchronized HCT-116 cells in early S-phase using aphidicolin and subsequently released the cells into MLN4924 or vehicle control containing media. Analysis of collected cells by flow cytometry indicated that control cells progressed normally through S-phase and mitosis and by 16 hours had lost their synchrony (Fig. 2A). In contrast, cells released into MLN4924 accumulated in S-phase as early as 4 hours after release from aphidicolin (Fig. 2A) and continued to increase DNA content (>4N) over the time course tested (Fig. 2A). Consistent with these cells being blocked in S-phase was the absence of detectable pH3, demonstrating a failure to enter mitosis (Fig. 2B). The cell cycle profile observed was

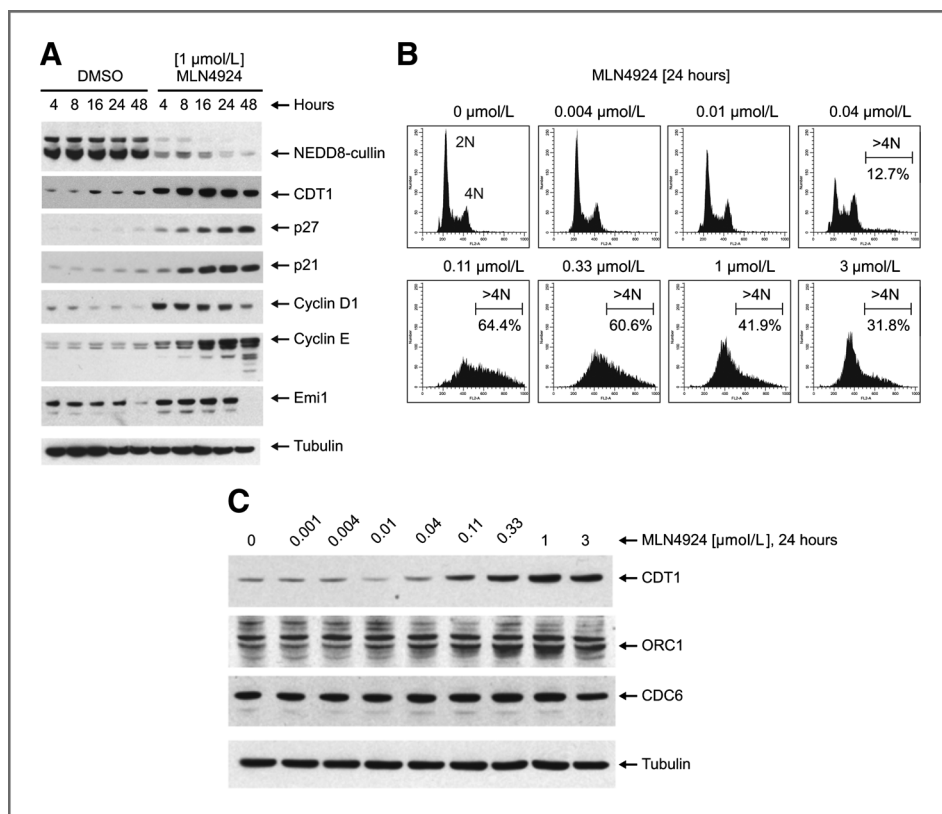


Figure 1. NAE inhibition results in deregulation of CRL substrates responsible for cell cycle progression causing S-phase defects. **A**, HCT-116 cells were treated with either DMSO or 1 $\mu\text{mol/L}$ MLN4924 for 4, 8, 16, 24, and 48 hours. Western blots were probed for NEDD8-cullin, CDT1, p27, p21, Cyclin D1, Cyclin E, Emi1, and tubulin. **B**, HCT-116 cells were treated with increasing concentrations of MLN4924 (0.004–3 $\mu\text{mol/L}$) for 24 hours. Treated cells were fixed and analyzed for cell cycle distribution by flow cytometry. More than 4N represents percentage of cells having greater than tetraploid DNA content. **C**, HCT-116 cells were treated with increasing concentrations of MLN4924 (0.001–3 $\mu\text{mol/L}$) for 24 hours and levels of CDT1, ORC1, CDC6, and tubulin were determined by Western blot analysis.

consistent with that previously detected on asynchronous HCT-116 cells treated with MLN4924 (Fig. 1B).

We observed normal modulation of CDT1 protein levels following release from synchronization into DMSO with low protein levels detected as cells progressed through S-phase (2–6 hours) which increased as cells exited mitosis (8–10 hours, Fig. 2B). In contrast, cells released into MLN4924 showed an increase in CDT1 protein with the expected concomitant loss of NEDD8-cullin conjugates, while accumulating in S-phase (Fig. 2B; refs. 19, 23). A parallel synchronization experiment was conducted using nocodazole; arresting cells in mitosis (see Fig. S3 in the Supplementary Material). In this experiment, cells released into MLN4924 progressed through mitosis, into G1 and entered S-phase while increasing their DNA content more than 4N without reentering mitosis. These results are consistent with our previous observations and confirmed that NAE inhibition primarily affects cells in S-phase.

To ascertain the levels of DNA synthesis in samples released from the aphidicolin block, cells treated with MLN4924 were labeled with BrdUrd prior to harvesting. After release, the DMSO-treated cells entered S-phase and the majority (90%) of the cells stained positive for BrdUrd (Fig. 2C). BrdUrd incorporation occurred in only 30% of cells after 6 hours as cells exited S-phase and subsequently increased at later timepoints consistent with a resumption of normal cell cycle progression. Cells released into MLN4924 also showed active BrdUrd incorporation ($\sim 90\%$) initially; however, in contrast to the

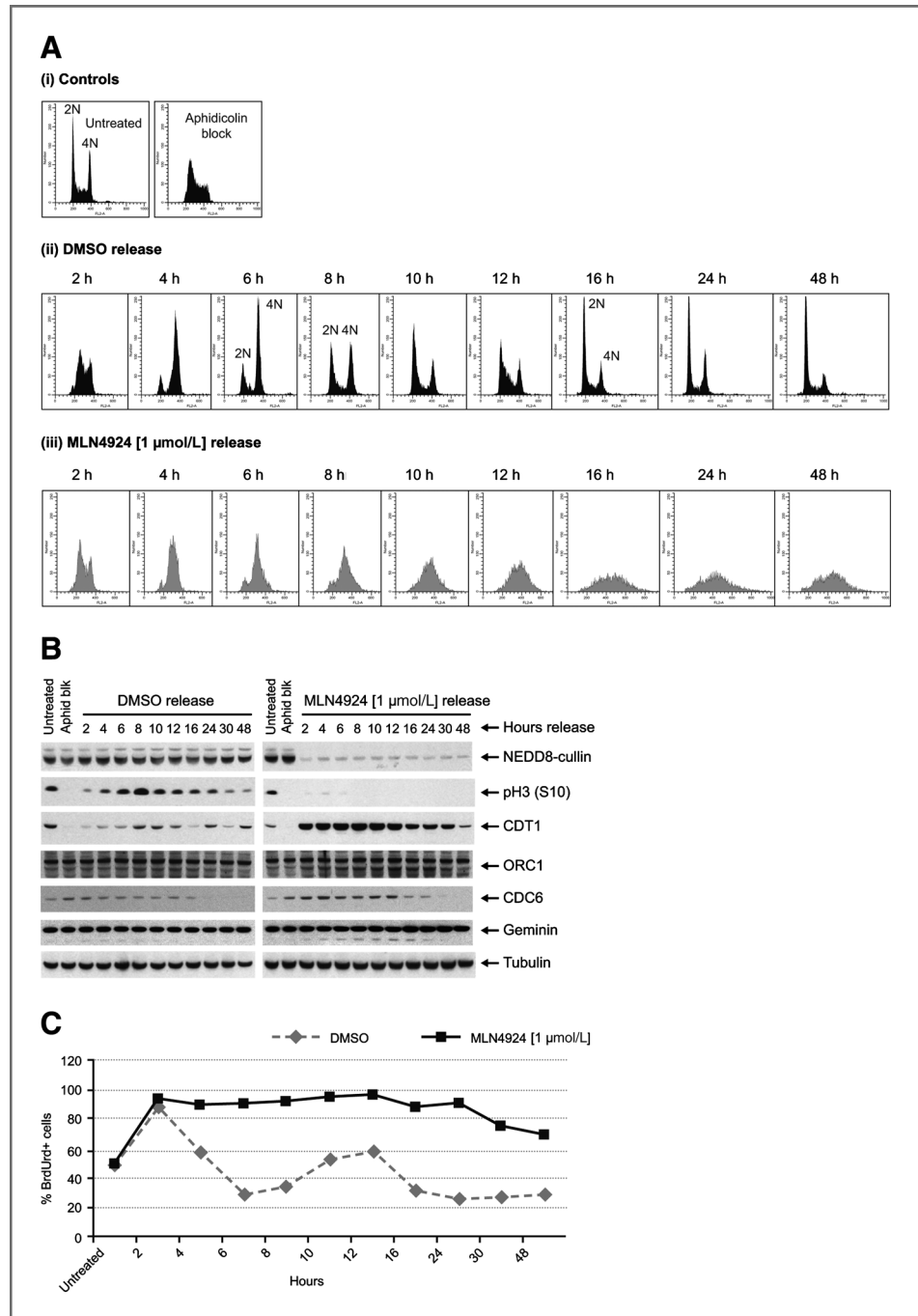
DMSO-treated cells, DNA synthesis was sustained through 48 hours postrelease (Fig. 2C). The failure of the MLN4924-treated cells to enter mitosis suggested that the abnormal increase in DNA synthesis was taking place within the same cell cycle, consistent with induction of DNA rereplication (22).

NAE inhibition induces DNA damage signaling and apoptosis consistent with rereplication

Overexpression of CDT1 or the ablation of its endogenous inhibitor, geminin, induces DNA rereplication and results in DNA damage leading to apoptosis (20, 22). We have shown that inhibiting NAE function in HCT-116 cells with MLN4924 activates a DNA damage checkpoint response, as marked by increasing levels of phospho-CHK1 (Ser317; pCHK1; refs. 15, 35). Several reports have outlined a sequential response to the DNA damage initiated by rereplication, which includes both single- and double-strand DNA breaks activating the ATR (ataxia telangiectasia and Rad-3-related) and ATM (ataxia telangiectasia mutated) kinase repair pathways (24, 36).

To elucidate whether the DNA damage response elicited by MLN4924 is a downstream consequence of CDT1 stabilization and rereplication, we followed the status of proteins known to be involved in signaling pathways activated by single- and double-strand DNA break repair. We reanalyzed samples from the aphidicolin synchronization block and release experiment and observed the expected immuno-staining for pCHK1 in response to the aphidicolin block (Fig. 3A; refs. 37, 38). The pCHK1 signal was lost by 4 hours in cells released into DMSO

Figure 2. NAE inhibition prevents progression through S-phase by deregulating CDT1 leading to DNA rereplication. **A**, HCT-116 cells were blocked at the G1/S transition and released from the block into either DMSO (control) or 1 $\mu\text{mol/L}$ MLN4924. At times indicated cells were collected, fixed, and analyzed by flow cytometry. **B**, HCT-116 cells were treated as in **A** and Western blot analysis performed to assess levels of NEDD8-cullin, p H3 (S10), CDT1, ORC1, CDC6, Geminin, and tubulin. **C**, HCT-116 cells were treated as in **A** and labeled with 10 $\mu\text{mol/L}$ BrdUrd for the final 30 minutes prior to harvest. Cells were collected, fixed, permeabilized, and stained with anti-BrdUrd antibody. Samples were analyzed by flow cytometry and the percentage of cells staining positive for BrdUrd quantified.



(Fig. 3A). In contrast, cells released into media containing MLN4924 initially recovered from the insult (4 hours), but by 6 hours the levels of pCHK1 were increasing again and remained elevated out to 24 hours, suggesting the induction of DNA damage and activation of the ATR kinase pathway was a result of MLN4924 treatment (Fig. 3A). Moreover, phosphorylation of RAD17 (S645; pRAD17) was also observed, which is consistent with recruitment of the RAD17/RFC and 9-1-1 repair complexes to the site of single-stranded DNA damage (24, 37).

The levels of the ATM pathway marker, phospho-CHK2 (T68) and the double-strand DNA breakage markers phospho-H2AX (S139; p H2AX) and phospho-p95/NBS1 (S343) also increased in cells released into MLN4924 (Fig. 3B). However, the accumulation of these markers occurred at much later time points (12–16 hours) compared to the ATR pathway markers (4–6 hours). The accumulation of these ATM and double-strand DNA damage markers continued out to 48 hours and coincided with a decrease in the phosphorylation

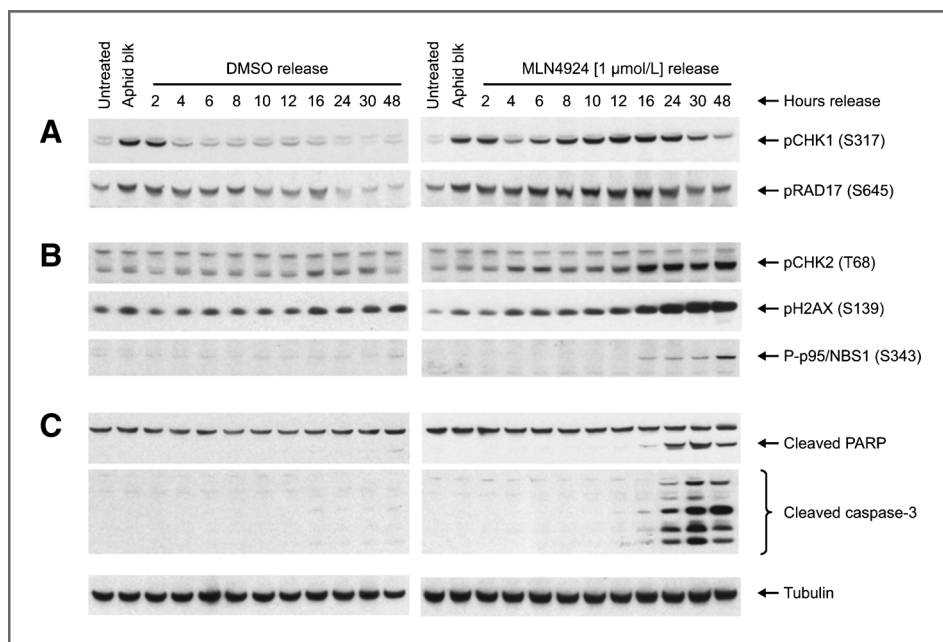


Figure 3. DNA rereplication induced by NAE inhibition results in DNA damage and apoptosis. HCT-116 cells were treated as in Figure 2A and Western blot analysis performed to detect markers of (A) ATR signaling [pCHK1 (S317) and pRAD17 (S645)], (B) ATM signaling [pCHK2 (T68), pH2AX (S139), and pNBS1 (S343)], and (C) apoptosis (cleaved PARP and cleaved caspase-3).

of CHK1 and RAD17, suggesting a transition from single-strand DNA damage to the accumulation of double-strand DNA breaks (Fig. 3A and B). Induction of the apoptotic markers cleaved PARP and cleaved caspase-3 in the MLN4924-treated samples appeared to correlate with activation of the double-strand DNA response pathway (Fig. 3C). Immunofluorescence staining of CDT1, pCHK1, pH2AX, and cleaved-caspase-3 confirmed that the sequential nature of these events was occurring uniformly across the treated population (see Fig. S4 in the Supplementary Material).

Knockdown of CDT1 suppresses rereplication in the presence of MLN4924

To investigate whether the rereplication phenotype observed following NAE inhibition by MLN4924 was mediated by CDT1 stabilization, we knocked down levels of CDT1 in HCT-116 cells using siRNA and monitored the ability of the cells to synthesize DNA in response to MLN4924. Effective knockdown of CDT1 was achieved and only modest accumulation of CDT1 was observed in these cells following MLN4924 treatment, presumably due to incomplete CDT1 protein depletion [Fig. 4A(i), lanes 3 and 4]. The control transfected sample treated with MLN4924 had the expected increase in CDT1 protein [Fig. 4A(i), lane 2]. Additional CRL substrates NRF2 and p27 (15) accumulated in both the GL2 and CDT1 RNAi-transfected samples when treated with MLN4924 as expected [Fig. 4a(i), lanes 2 and 4]. BrdUrd incorporation was significantly reduced in cells lacking CDT1 following 48 hours of siRNA treatment in the presence or absence of MLN4924 compared to control cells (Fig. 4B). In these samples 77% of the GL2 RNAi-transfected control cells were synthesizing DNA following treatment with MLN4924, compared to only 28% of the CDT1 RNAi-transfected cells, suggesting that accumulation of CDT1 following MLN4924 treatment medi-

ates the rereplication phenotype (see Fig. S5 in the Supplementary Material for FACS histograms). Moreover, DNA damage activation (pCHK1) was not observed in the CDT1 RNAi-transfected cells following MLN4924 treatment [Fig. 4A(ii), lanes 3 and 4], compared to GL2-transfected cells treated with MLN4924 [Fig. 4A(ii), lane 2]. These data suggest that following NAE inhibition, the induction of rereplication and DNA damage observed correlates to the stabilization of CDT1 demonstrating its critical role in the response.

Cullin knockdown stabilizes CDT1 and recapitulates the rereplication phenotype

Depending on the cellular context, CRL1^{Skp2} and/or CRL4-DDB1^{CDT2} can facilitate the ubiquitination and proteasomal degradation of CDT1 (32). Silencing of DDB1 and CDT2 by siRNA has been shown to result in the accumulation of CDT1 and lead to rereplication (39). To confirm that inhibition of cullin 1 (CUL1) and cullin 4 (CUL4a/b) neddylation by MLN4924 results in CDT1 elevation and rereplication we measured CDT1 levels and cell cycle distributions following siRNA depletion of CUL1 and/or CUL4a/b. Interestingly, CUL1 or CUL4a/b depletion alone resulted in little elevation of CDT1 at 48 hours even though a modest increase in more than 4N DNA content was observed (Fig. 5). This may be the result of a transient elevation of CDT1 initiating rereplication that persisted even when alternative mechanisms of CDT1 turnover have reduced CDT1 protein to baseline levels (40) or from a small subset of the population that was untransfected. The combined knockdown of CUL1 and CUL4a/b resulted in accumulation of CDT1 and the substantial rereplication phenotype comparable to MLN4924 treatment [Fig. 5A(ii) and B]. These data add to the reports of overlapping function of CUL1 and CUL4a/b in controlling CDT1 turnover following replication initiation. To further define the role of CDT1 in initiating

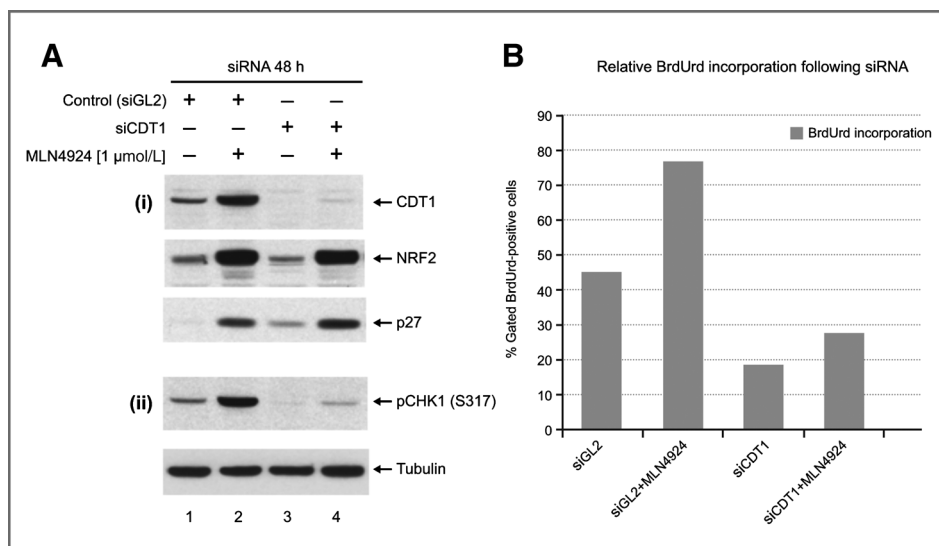


Figure 4. CDT1 RNAi rescues the DNA rereplication phenotype upon NAE inhibition. **A**, HCT-116 cells were transfected with CDT1 or GL2 (control) siRNAs for 48 hours. MLN4924 (1 $\mu\text{mol/L}$) was added for the final 16 hours prior to harvest and relevant pathway markers were analyzed by Western blot [CDT1, NRF2, p27, pCHK1 (S317), and tubulin]. **B**, HCT-116 cells were transfected with CDT1 or GL2 (control) siRNAs for 48 hours, 1 $\mu\text{mol/L}$ MLN4924 was added during the final 16 hours as indicated, and all samples were pulse labeled with 10 $\mu\text{mol/L}$ BrdUrd for 30 minutes. Cells were collected, fixed, permeabilized, and stained with anti-BrdUrd antibody. Samples were analyzed by flow cytometry and the percentage of cells staining positive for BrdUrd quantified.

these events we depleted CDT1 along with CUL1 and CUL4a/b in cotransfection experiments (see Fig. S6 in the Supplementary Material) and confirmed a reduction in cells having more than 4N DNA content. A small population of cells with more than 4N DNA content was still detectable, likely due to incomplete knockdown of CDT1 (as observed in Fig. S6B, lanes 4, 6, and 8); however, we cannot completely exclude the possibility of other CRL substrates playing a role in the phenotype.

Effects of NAE inhibition on cycling and noncycling cells

Our results suggest that following NAE inhibition, the dominant phenotype leading to cell death is the induction of DNA rereplication and DNA damage as cells transition through S-phase. Therefore nonproliferating cells would fail to undergo DNA rereplication following MLN4924 treatment. To illustrate this, MCF10A cells were treated with MLN4924 for 24 hours under conditions in which the cells were cycling (subconfluent) or noncycling (confluent, contact inhibited), and compared to actively cycling HCT-116 cells. Western blot analysis confirmed reduced NEDD8-cullin levels, indicating NEDD8 pathway inhibition under all growth conditions (Fig. 6A, lanes 2, 3, 5, 6, 8, and 9); however, accumulation of CDT1 and induction of DNA damage (pCHK1 activation) were only observed in the cycling cells (Fig. 6A, lanes 2, 3, 8, and 9). Moreover, flow cytometry analysis of cycling and noncycling MCF10A cells treated with 1 $\mu\text{mol/L}$ MLN4924 for 24 hours showed that only the cycling cells demonstrated a rereplication cell cycle distribution similar to HCT-116 cells treated with MLN4924 (Fig. 6B). This suggests that proliferating cells

are more sensitive to the DNA rereplication phenotype generated following NAE inhibition.

Discussion

In this report we characterize the cellular effects of specifically inhibiting NAE using the small molecule MLN4924. Inhibition of NAE results in cell death in human tumor cells and our data support a model for the primary cellular mechanism of action as induction of DNA rereplication (Fig. 7). Our model suggests loss of NAE function prevents the proper CRL-dependent regulation of CDT1, thereby leading to the induction of DNA rereplication and subsequent DNA damage. The initial engagement of the ATR repair pathway occurs in response to single-strand DNA breaks, resulting in checkpoint activation designed to arrest cells for DNA repair. The persistent rereplication observed eventually leads to double-strand breaks, as demonstrated by phosphorylation of proteins downstream of the ATM repair pathway. The inability of cells to repair the DNA damage ultimately leads to induction of apoptosis and cell death. It is noteworthy that similar cell cycle effects were observed when NAE activity was reduced in a temperature-sensitive mutant hamster cell line as well as through knockdown of NAE by siRNA in HCT-116 cells (15, 41).

NAE inhibition effectively shuts down CRL activity and deregulates the turnover of many proteins within the cell (15). Unexpectedly, our data suggest that CDT1 stabilization appears to be a critical factor leading to the observed rereplication outcome (22, 42). The inability to properly degrade CDT1 in S-phase of the cell cycle has been shown to result in

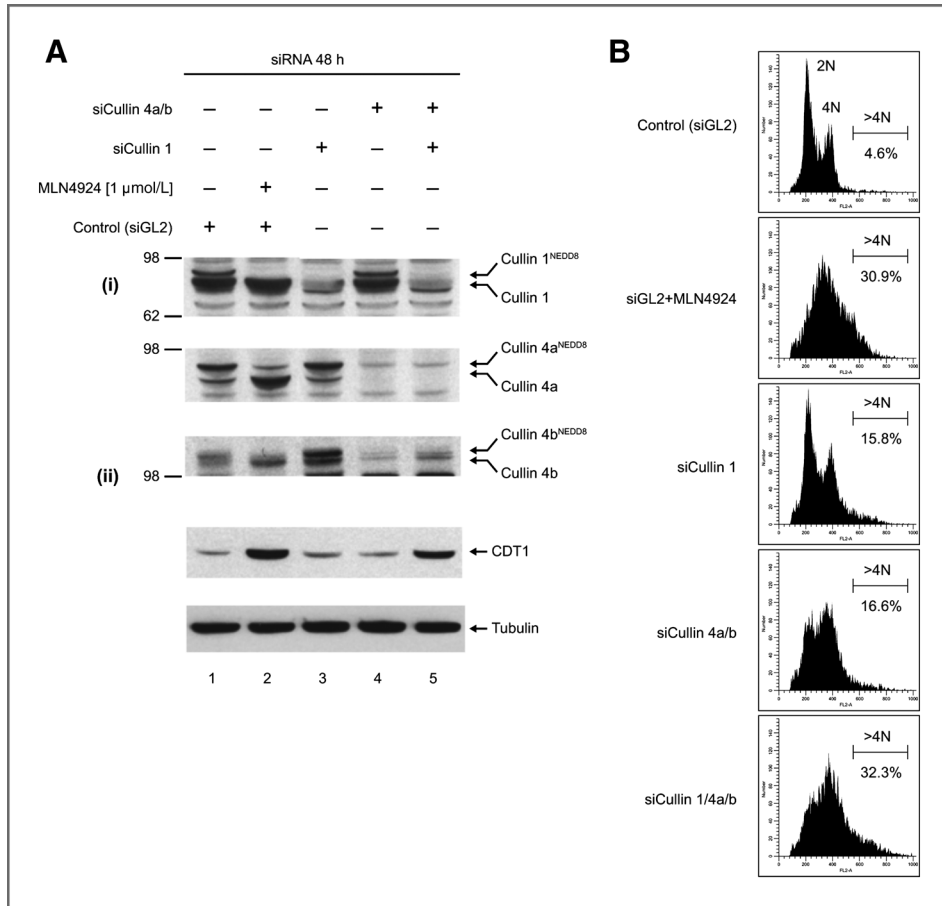


Figure 5. Cullin 1 and 4A/B depletion stabilizes CDT1 and recapitulates the rereplication phenotype observed following NAE inhibition. A, HCT-116 cells were transfected with Cullin 1, Cullin 4A/B, and GL2 (control) siRNAs for 48 hours. MLN4924 (1 μmol/L) was added for the final 16 hours prior to harvest and levels of Cullin 1, Cullin 4A, Cullin 4B, and CDT1 determined by Western blot analysis. B, HCT-116 cells were transfected as in A and collected, fixed, and analyzed by flow cytometry. More than 4N represents percentage of cells having greater than tetraploid DNA content.

DNA rereplication (19, 23, 43). These observations are also consistent with overexpression of CDT1 as well as siRNA knockdown of its endogenous inhibitor, geminin, in cultured cells (20, 22, 44). Geminin prevents rereplication through

binding to CDT1 and maintaining it in an inactive form and siRNA reduction of geminin levels has been shown to induce DNA rereplication (20, 23, 44, 45). Since MLN4924 does not affect geminin levels, the increased rereplication following

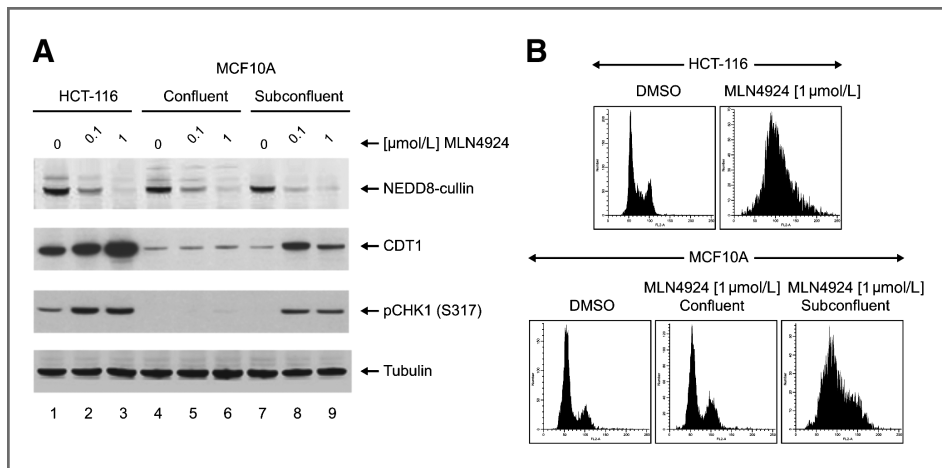


Figure 6. Proliferating cells are more sensitive to DNA rereplication affects following NAE inhibition than nonproliferating cells. HCT-116 cells plated at 5×10^5 cells/mL, and MCF10A cells plated at 5×10^5 cells/mL (proliferating) and 2×10^6 cells/mL (nonproliferating), were treated with 1 μmol/L MLN4924 for 24 hours. A, Western blots were probed for NEDD8-cullin, CDT1, pCHK1 (S317), and tubulin. B, samples were analyzed by flow cytometry for cell cycle distribution.

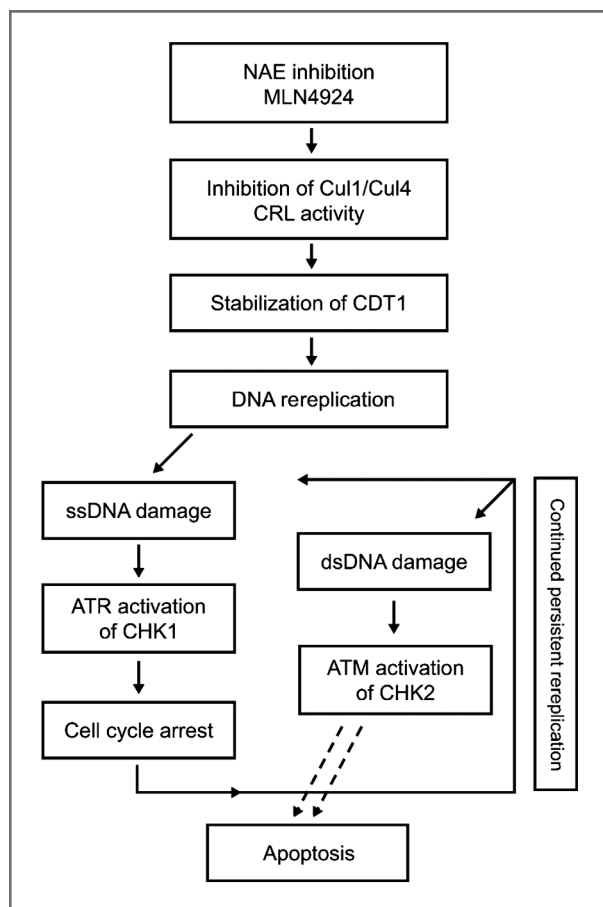


Figure 7. Proposed model for induction of DNA rereplication following NAE inhibition with MLN4924. NAE inhibition prevents the proper CRL-dependent turnover of the preRC component CDT1, which induces DNA rereplication as the cells progress through S-phase. Resulting DNA damage activates the ATR/CHK1 pathway, and prevents further cell cycle progression; in turn, persistent rereplication then signals the ATM/CHK2 pathway. Failure to repair damaged DNA ultimately leads to activation of apoptosis and loss of cell viability. Model adapted with permission from J. J. Lin and A. Dutta (24).

NAE inhibition is likely not due to deregulation of geminin (ref. 15; Fig. 2B). Although additional components of the pre-RC complex, ORC1 and CDC6, were evaluated, our results showed only minimal effects from NAE inhibition on their cellular protein levels.

To confirm the critical role of CDT1 in the induction of rereplication following MLN4924 treatment, we sought to rescue the phenotype by knocking down CDT1 levels with siRNA. Following CDT1 knockdown, cells incorporate less BrdUrd even in the presence of MLN4924 indicating a suppression of DNA synthesis and rereplication, suggesting a key role of CDT1 in the phenotype. Noncycling MCF10A cells, when treated with MLN4924, did not undergo rereplication and failed to stabilize CDT1 compared to cycling MCF10A cells, supporting our findings (see Fig. 6). Based on these data, cells must progress through S-phase in the presence of

MLN4924 to be deleteriously affected, suggesting that proliferating cancer cells may be more sensitive to NAE inhibition compared to quiescent cells.

Since there are at least 2 CRLs responsible for CDT1 degradation (CUL1 and CUL4), we sought to determine the importance of each in regulating CDT1 levels in HCT-116 cells (32, 42, 43). We demonstrated that only the combined siRNA-mediated knockdown of CUL1 and CUL4a/b was sufficient to stabilize CDT1 and elicit the rereplication phenotype. Knockdown of CUL1 or CUL4a/b alone yielded only a modest change in cell cycle distribution without an apparent stabilization of CDT1 supporting their overlapping functions in regulating the turnover of CDT1 (19). Cotransfection experiments knocking down CDT1 along with CUL1 and/or CUL4 reduced the population of cells having more than 4N DNA content, supporting a primary role of CDT1 in the observed rereplication phenotype. However, a small fraction of cells with more than 4N DNA content was still evident suggesting that other, yet to be defined, CRL substrates may also play a role in the rereplication response. Interestingly, we observed that CUL4 appeared to be approximately 90% neddylated compared to approximately 10% for CUL1 which suggest a hierarchy of control of CDT1 turnover within the CUL1/CUL4 axis. Thus, the neddylation status of CUL4 and/or CUL1 in different cell types may play a role in predicting sensitivity or magnitude of response to MLN4924-induced DNA rereplication and warrants further investigation.

A major consequence of DNA rereplication is DNA damage and activation of checkpoint signaling (20, 22, 46–48). Previous studies have reported that the ATR/CHK1 kinase pathway is activated in response to rereplication and phosphorylation of RAD17 occurs at sites of damaged DNA following rereplication (24, 37, 39). Our results show that both CHK1 and RAD17 are phosphorylated in response to MLN4924 treatment, suggesting that the cells undergo single-strand DNA damage following rereplication, consistent with accumulation of damaged replication forks (37, 49). CHK1 and RAD17 phosphorylation is sustained for at least 30 hours following induction of rereplication during which time cells continue to synthesize DNA. The fact that cells continue to incorporate BrdUrd suggests that MLN4924 may in some way interfere with CRL substrates which regulate the intra-S-phase checkpoint, and this may warrant further investigation (49, 50).

In the absence of NAE function cells no longer enter mitosis and are not capable of properly regulating DNA replication. The induction of rereplication following siRNA knockdown of geminin has been shown to activate the ATM/CHK2 kinase and MRE11-RAD50-NBS1 (MRN) complexes (24, 36). The activation of the ATR/CHK1 pathway occurs upstream of the ATM/CHK2 pathway and appears to be required for its activation (24, 39), and see model in Fig. 7). Consistent with these observations, we detected the appearance of markers of double-strand DNA damage and ATM repair pathway activation (pCHK2, p2AX, and pNBS1) following ATR activation. These observations illustrate a switch from the accumulation of single-strand DNA damage to double-strand DNA damage and support a sequential response to DNA rereplication consistent with published reports (24). The appearance of

markers of apoptosis correlates with the markers of double-strand DNA damage, suggesting that cell death is occurring because of accumulated double-strand DNA damage (see model in Fig. 7). Interestingly, p53 was phosphorylated (Ser15) in response to MLN4924 which may suggest a role of p53 in the rereplication response. However we observed a similar cellular phenotype of NAE inhibition in a panel of cells irrespective of their p53 status (see Fig. S7 in the Supplementary Material; ref. 44). Further work is required to understand if there is a specific genetic background that may make cells particularly sensitive to NAE-mediated DNA rereplication and cell death.

In this report we outline an unexpected cellular mechanism of action for MLN4924 (and NAE inhibition) in various human tumor cell lines in which the deregulation of CDT1, a key CRL substrate, leads to DNA rereplication. It will be important to further define the mechanisms involved in the cellular response to rereplication, how rereplication affects cell sensitivity, and how this may impact the potential mechanism(s) of resistance to MLN4924. Based on our data we hypothesize that cells that have defective DNA damage repair pathways (e.g. BRCA1 mutants, Fanconi Anemia) might be more sensitive to MLN4924 than cells with functional DNA repair pathways (48). In addition, combination of MLN4924 with known DNA damaging and/or S-phase-inducing agents, as well as inhibitors of

DNA repair pathways, could be a way to further sensitize cells to MLN4924 treatment. To this end, a genome wide RNAi screen using MLN4924 is in process to support rational drug combinations and, interestingly, it appears that MLN4924 has a profile that is distinct from other FDA-approved DNA damaging agents (E. Lightcap, manuscript in preparation). Further understanding of this mechanism and defining the types of DNA damage and/or damage repair pathways involved in the MLN4924 response will be required as MLN4924 progresses through clinical development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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