The NEDD8 inhibitor MLN4924 increases the size of the nucleolus and activates p53 through the ribosomal-Mdm2 pathway

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INTRODUCTION

The NEDD8 ubiquitin-like molecule is essential for viability, growth and development, and is a potential target for therapeutic intervention. We found that the small molecule inhibitor of NEDDylation, MLN4924, alters the morphology and increases the surface size of the nucleolus in human and germline cells of Caenorhabditis elegans in the absence of nucleolar fragmentation. SILAC proteomics and monitoring of rRNA production, processing and ribosome profiling shows that MLN4924 changes the composition of the nucleolar proteome but does not inhibit RNA Pol I transcription. Further analysis demonstrates that MLN4924 activates the p53 tumour suppressor through the RPL11/RPL5-Mdm2 pathway, with characteristics of nucleolar stress. The study identifies the nucleolus as a target of inhibitors of NEDDylation and provides a mechanism for p53 activation upon NEDD8 inhibition. It also indicates that targeting the nucleolar proteome without affecting nucleolar transcription initiates the required signalling events for the control of cell cycle regulators.

Received 17 October 2014; revised 10 February 2015; accepted 3 March 2015

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www.nature.com/onc
a co-operation between the two RPs and 5S rRNA enhances Mdm2 inhibition under ribosomal stress conditions.\textsuperscript{26,27} This causes p53 stabilization, activation of the p53 response and cell cycle arrest.\textsuperscript{28–31}

We found that inhibition of NEDDylation by MLN4924 alters the morphology and increases the surface size of the nucleolus in the absence of nucleolar fragmentation both in human cells and in \textit{Caenorhabditis elegans} germline cells. Combination of SILAC quantitative proteomics, rRNA production/processing and ribosome profiling show that in contrast to the established nucleolar stressor ActD, MLN4924 alters the nucleolar proteome but does not affect RNA Pol I transcription. Further analysis shows that MLN4924 activates the p53 pathway through the RP-Mdm2 module with characteristics of nucleolar stress. This study reveals that MLN4924 causes activation of key tumour suppressor pathways through the nucleolus. Furthermore, the data support a conserved role for NEDDylation as a regulator of nucleolar signalling. As MLN4924 is currently being tested in clinical trials, this study identifies the nucleolus as a target for the action of a potential novel chemotherapeutic drug.

**RESULTS**

MLN4924 increases the surface size of the nucleolus in human cells and in \textit{C. elegans} germline cells

Inhibition of NEDDylation by MLN4924 produces diverse biological effects mainly through inhibition of CRL function. We observed that treatment with MLN4924 altered the morphology of the nucleolus without detectable nucleolar fragmentation (Figure 1a).

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**Figure 1.** (a) H1299 cells were treated either with DMSO (control) or with MLN4924 (1 μM) for 24 h. Phase contrast micrographs were then taken and arrows indicate the position of nucleoli. (b) U2OS cells were treated with DMSO or MLN4924 (1 μM, 15 h) before staining for nucleolin, fibrillarin or B23. (c) U2OS cells were treated as above for 24 h before cells were fixed and stained for fibrillarin to define the nucleolus and with DAPI to define the nucleus. The relative surface of the nucleoli was determined as described in Supplementary Information. (d) Quantitation of data represented as relative changes in surface size of the nucleolus (nucleolus/nucleus). ***P < 0.001. (e) U2OS cells were treated with DMSO, ActD (5 nM) or doxorubicin (1 μM) for 24 h before fixing and staining for fibrillarin as in (a, c).
organization, we monitored the effect of MLN4924 on the localization of nucleolar proteins that reside within distinct areas in the nucleolus. U2OS cells were either untreated or treated with MLN4924, and the localization of nucleolin and fibrillarin (dense fibrillar component) and B23/nucleophosmin (granular component) was monitored. MLN4924 has no effect on the localization of any of the tested nucleolar proteins, confirming that MLN4924 affects the morphology but not the integrity of the nucleolus (Figure 1b). We also observed that the surface size of the nucleolus is altered in the MLN4924-treated compared with untreated cells. We designed an experiment, similar to that described by Nicholas Baker,32 to quantify changes in the nucleolar surface size under different conditions. We performed fibrillarin and DAPI staining to define the nucleolus and the nucleus area, respectively, using the same cross-sectional areas. To provide a measure of nucleolar and nuclear surface area, we determined the number of pixels by defining the minimal and maximal thresholds independent of labelling intensity, as described in Supplementary Information. The ratio of the nucleolar to nuclear area determines the relative changes in nucleolar size under different conditions. MLN4924 caused a significant increase in nucleolar surface area compared with control cells (Figures 1c and d). As controls we used the chemotherapeutic drugs ActD and doxorubicin that cause nucleolar segregation (Figure 1e). Protein NEDDylation is a highly conserved posttranslational modification. We used C. elegans as the model organism to determine the conservation of our observations on the effect of NEDD8 inhibition on nucleolar morphology and to also establish the physiology of our observations at an organism level. We treated C. elegans with MLN4924 and performed similar analysis on the surface area of the nucleolus to that performed in human cells.33 We focused on germline cells as they rapidly proliferate and contain relatively large nucleoli that can be easily monitored and studied. Animals were either untreated or treated with MLN4924, and germline cells were dissected and stained for fibrillarin and with DAPI to determine the nucleolar and nuclear area as before. We found that similarly to what is observed in human cells, MLN4924 caused a significant increase in nucleolar surface area (Figures 2a and b). Similar results were obtained with siRNA against the C. elegans NEDD8 (NED-8) (Figures 2a and b). Application of genotoxic treatments including UV, ActD or doxorubicin caused the formation of either distinct nucleolar fragments (UV) or subfragmentation (segregation) within the defined nucleolar area (ActD and doxorubicin) (Figure 2c). The above data identify a role for NEDD8 in controlling nucleolar morphology and surface area, which is conserved between humans and C. elegans.

![Image](https://example.com/image.png)

**Figure 2.** (a) C. elegans L4 adults were treated with DMSO or with MLN4924 (100 μM for 24 h) or fed with NED-8 RNAi bacteria. The germlines were then dissected and stained for fibrillarin and DAPI as in Figure 1. (b) The surface size of the nucleolus was determined as in Figure 1d. ***P < 0.001. (c) in the experiment performed in a, animals were also treated with 100 J/m², or ActD (100 nM) or doxorubicin (100 μM) as described in Materials and methods.
MLN4924 activates p53 through the RP-Mdm2 pathway
Changes in the nucleolar morphology usually reflect changes in the nucleolar function, which are detected by multiple cell cycle regulators. The p53 tumour suppressor is a sensitive sensor of nucleolar misfunction. Ribosomal proteins are key players in transmitting defects in nucleolar function to p53 through inhibition of the Mdm2 E3-ligase. We hypothesized that if p53 activation by MLN4924 is due to the observed changes in nucleolar morphology, it should depend on the RP-Mdm2 signalling pathway. Further evidence to support this hypothesis derive from previous studies that showed that inhibition of NEDDylation of RPL11 participates in p53 activation upon nucleolar stress.

MCF7 cells, which contain wild-type endogenous p53 were treated with increasing doses of MLN4924. As expected, MLN4924 decreased Cullin-1 NEDDylation (Figure 3a). MLN4924 also increased p53 levels in a dose-dependent manner and the expression of p53-regulated genes, consistent with previous studies and with the notion that inhibition of NEDDylation causes p53 activation (Figure 3a). Similar effects of MLN4924 were observed in other p53-positive cell lines, including U2OS, HCT116 and A375 (data not shown).

We next determined the effect of MLN4924 on L11 NEDDylation. H1299 cells were transiently transfected with plasmid vectors encoding Flag-L11 and His6-NEDD8. His6-NEDDylated proteins were isolated by Ni-agarose chromatography and blotted for Flag-L11. MLN4924 decreased RPL11 NEDDylation (pull-down) relative to total levels of RPL11 (whole extracts) (Figure 3b). Consistent with the notion that prolonged inhibition of NEDDylation also leads to RPL11 degradation, MLN4924 decreased the total levels of RPL11 (Figure 3b).

The nucleolus is a dynamic structure, and it has been established that nucleolar disruption correlates with altered mobility kinetics of many nucleolar proteins. We used live cell imaging and fluorescence recovery after photobleaching to determine the effect of MLN4924 on the mobility of RPL11. We used H1299 cells stably expressing RPL11-EGFP that were either untreated or treated with MLN4924. As control, we used ActD, which is known to affect the mobility of ribosomal proteins. By measuring in real time the recovery of the EGFP signal in the nucleolus after bleaching, we found that both MLN4924 and ActD decreased the mobility of RPL11-EGFP (Figure 3c).

The data suggest that MLN4924 may impact on p53 function through mechanisms involving RPL11. To determine the role of RPL11 in p53 stabilization by MLN4924, we transfected MCF7 cells

Figure 3. (a) MCF7 cells were treated with MLN4924 at the indicated doses for 24 h. Cells were lysed in 2× SDS buffer and extracts were analysed by western blotting. Equal loading was monitored with β-actin. (b) H1299 cells were transfected with His6-NEDD8 (2 µg), Flag-L11 (5 µg). Twenty-four hours post transfection, cells were treated with MLN4924 (1 µM) for the indicated times before cells were harvested. NEDDylated proteins and total cell extracts were prepared as described in Material and methods and analysed by western blotting with the appropriate antibodies. Signals for NEDD8-L11 and total L11 were quantified using Image Gauge. Changes in absolute L11-NEDDylation upon MLN4924 treatment were determined by the ratio of NEDDylated over total L11. (c) H1299 cells stably expressing RPL11-EGFP were treated for 4 h with DMSO, ActD (5 nM) or MLN4924 (1 µM) before the fluorescence recovery after photobleaching experiment. The half-life of recovery (bottom left) and the mobile fraction (bottom right) were calculated as described in materials and methods.

either with control or RPL11-specific siRNAs and treated with either MLN4924, or with low doses of ActD. MLN4924 causes p53 stabilization and induction of the well-described p53-regulated gene, Mdm2 (Figures 4a left and 4b top panel).\textsuperscript{35} However, knockdown of RPL11 reduced p53 stabilization and transcriptional activation by MLN4924, similar to that observed with ActD (Figures 4a and b). The defects in p53 stabilization by MLN4924 were observed with four separate siRNAs, targeting different parts of the RPL11 mRNA sequence (Figure 4c). Therefore, RPL11 is required for p53 activation by MLN4924, similar to what is observed by ActD-induced nucleolar stress.\textsuperscript{40,41}

The Mdm2 E3 ligase is an important p53 regulator, and is known to play a critical role in p53 activation upon nucleolar stress.\textsuperscript{30,42} Direct binding of RPs to Mdm2 blocks Mdm2-mediated p53 degradation allowing p53 accumulation.\textsuperscript{43} For RPL11 and RPL5, genetic studies demonstrated the importance of their

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Figure 4. (a) MCF7 cells were transfected with either control or RPL11 siRNAs in duplicates, before treatment with MLN4924 (1 μM, left panel) or ActD (5 nM, right panel) as indicated. Cell extracts and western blot analysis was performed as in Figure 1. (b) Experiment performed as in (a) for the indicated time points and mRNA was isolated as described in Materials and methods. Expression of the mdm2 gene was monitored by qPCR. Expression of actin was used to normalize the values. Data are represented as mean values +/- s.d. from three independent experiments. (c) Experiment performed as in (a) with individual siRNAs against different parts of RPL11 sequence and treating cells with MLN4924 as indicated. (d) Wild-type MEFs or MEFs expressing the Mdm2C305F mutant were treated with ActD (5 nM) or MLN4924 (1 μM) as indicated. Cell extracts were analysed by western blotting for the indicated proteins. (e) *C. elegans* L4 adults were treated as indicated before expression of *egl-1* was determined by qPCR. For UV, expression was determined 5 h post treatment and 24 h post chemical treatment.
binding to Mdm2 in p53 nucleolar signalling. Transgenic mice expressing a single amino acid mutant of Mdm2 (C305F) that cannot interact with RPL11 and RPL5 were shown to be resistant to nucleolar stress induced by ActD. To test whether p53 activation by MLN4924 depends on RPL11/RPL5-Mdm2 interaction, we used mouse embryonic fibroblasts (MEFs) that express either wild-type Mdm2 or the Mdm2 C305F mutant. As reported, low doses of ActD cause p53 stabilization in the wild-type MEFs, but not in the Mdm2 C305F mutant MEFs (Figure 4d). MLN4924 stabilized p53 in wild-type MEFs, although to a much lesser extent than in other cell lines tested. However, in the Mdm2 mutant MEFs, MLN4924 did not cause any detectable p53 stabilization (Supplementary Information S1). In combination, the above data strongly suggest that MLN4924 causes p53 stabilization through the nucleolus involving the RP-Mdm2 pathway. We also tested the effect of MLN4924 in the activation of CEP-1, the p53 homologue in C. elegans, which lack both key p53 regulators Mdm2 and Mdmx. Treatment of C. elegans with either MLN4924 or ActD had no effect on CEP-1/p53 transcriptional activity (Figure 4e) despite the clear effects on nucleolus size and morphology (Figure 2c). In contrast, treatment with UV causes a robust CEP-1/p53 activation (Figure 4e). The data in C. elegans recapitulate the results in the transgenic Mdm2C305F mice where p35 is insensitive to nucleolar stress induced by ActD, but not to other forms of DNA damage, owing to lack of Mdm2-RP binding. Therefore, lack of nucleolar stress signalling to CEP-1/p53 in C. elegans may be due to the absence of Mdm2.

MLN4924 alters the composition of the nucleolar proteome but does not inhibit nucleolar transcription

Comparison between the effects of MLN4924 and ActD within the p53 pathway shows several similarities: both compounds activate p53 in RPL11/RPL5 and Mdm2C305F dependent manner, inhibit RPL11 NEDDylation and decrease RPL11 nucleolar mobility. The fact that at low doses (1–5 μM), ActD is relatively specific in blocking RNA Pol I-dependent transcription raises the possibility that MLN4924 can elicit its effect on p53 through transcriptional inhibition in the nucleolus.

We performed different experiments to determine the effect of MLN4924 on nucleolar function. We combined SILAC with mass spectrometry to provide a quantitative proteomic analysis of the nucleolus upon inhibition of NEDDylation. U2OS cells labeled with arginine and lysine containing either light (R0K0), medium (R6K4) or heavy (R10K8) stable isotopes were either untreated or treated with ActD (medium) or with MLN4924 (heavy) for 15 h. Equal numbers of light, medium and heavy cells were mixed, fractionated and nucleoli isolated (Figure 5a). Proteins from the resulting nucleolar extracts were separated by gel filtration and each fraction trypsin-digested and analysed by mass spectrometry (Supplementary Information S2). Over 1300 proteins were identified and quantified. Treatment with MLN4924 caused an increased abundance of 98 proteins and a decreased abundance of 21 nucleolar proteins by at least 1.3-fold relative to nucleolar proteins of untreated cells (Figure 5b and Supplementary Information), whereas low doses of ActD, resulted in 95 nucleolar proteins increasing in abundance and 31 proteins decreasing, by at least 1.5-fold relative to nucleolar protein levels in the untreated control cells (Figure 5b and Supplementary Information).

Network analysis for the regulated proteins did not provide any evidence for an effect of MLN4924 on RNA Pol I-dependent transcription. Rather, components of the RNA Pol II and DNA replication processes were significantly enriched in the nucleolus upon MLN4924 treatment. This includes elongation activators TCEB1, 2, the component of the core-TFIH transcription factor GTF2H1, multiple members of the MCM family of DNA replication licensing factors, along with histone acetyltransferase complexes including MRFAP1, MORF4L2 and MORF4L1, Tip60 and the histone de-acetylase HDAC1 (Figure 5c and Supplementary Information S3A and B). The nucleolar abundance of histone H1 and many of its variants was decreased by MLN4924 (Supplementary Information S3A and B). Histone H1 is preferentially associated with the 'linker' DNA between nucleosomes and is thought not to have a major effect on global transcription but can act as positive or negative gene-specific regulator of transcription in vivo. In contrast and as expected, the major targets of ActD are regulators of RNA Pol I transcriptional activity and RNA processing including coillin and fibrillarin (Supplementary Information S3C). A group of proteins affected both by MLN4924 and ActD was also identified (Figure 5d). Interestingly, the relative abundance of RRS1 (ribosome biogenesis regulatory protein) was decreased following treatment of cells with either compound, while other proteins, such as PCNA (proliferating cell nuclear antigen) and DNA replication licensing factors, were differentially affected, with MLN4924 increasing and ActD decreasing their nucleolar abundance, respectively. The data indicate that inhibition of NEDDylation causes changes in the nucleolus on a similar scale to the well-described chemotherapeutic drug ActD, but does not affect RNA Pol I activity. We used H1299 cells, which are genetically null for p53, to avoid the potential downstream effects of p53 activation on RNA Pol I transcription. Incorporation of 3H-uridine on nascent rRNA was monitored in H1299 cells treated either with low doses of ActD or with MLN4924. As expected, ActD caused a dramatic inhibition of 47S rRNA production and in the appearance of the 32S, 28S and 18S rRNA processed forms (Figure 5e). However, the application of MLN4924 did not cause any detectable changes in rRNA production (Figure 5e). Consistently, analysis of pre-rRNAs by northern blotting after MLN4924 treatment did not reveal any major differences for the 40S precursors (5′IT51 probe) compared with control cells, while ActD-treated cells were almost devoid of pre-rRNAs (Figure 5f). To further investigate the potential effect of MLN4924 on ribosome subunit biogenesis, we analysed ribosome production using sucrose gradient ultracentrifugation. As control, we used ActD, which impairs ribosome subunit biogenesis, mainly through inhibition of rRNA production. As expected, ActD caused a decrease in large and small ribosome subunits and polysome formation (Figure 5g). MLN4924 treatment had no detectable effect on the polysome fraction, but it caused a decrease in the ratio of 60S/40S subunit production (Figure 5g). Similar defects in the 60S/40S ratio have been reported upon knockdown of large ribosome subunit proteins RPL7a, RPL11, RPL5, RPL14, RPL26 and RPL35a. The combination of the data suggest that the activation of p53 by MLN4924 is not due to RNA Pol I inhibition but rather due to re-organization of the nucleolar proteome that involves targeting RPs.

Differential roles of RPL11 and p53 in MLN4924-induced accumulation of cells in S-phase and cytotoxicity

Our studies have identified RPL11 as an important cellular factor required for the MLN4924-induced p53 activation. The MLN4924-induced toxicity in proliferating cells is mainly due to the resulting S-phase arrest. We tested the effect of p53 and RPL11 knockdown on cell cycle progression (Figure 6a and Supplementary Information S4) and viability (Figure 6b) upon inhibition of NEDDylation by MLN4924. As expected, treatment of control siRNA-transfected MCF7 cells with MLN4924 increased the proportion of cells in S-phase with a corresponding decrease in cell viability (Figures 6a and b). Knockdown of p53 promoted the accumulation of cells in S-phase following MLN4924 treatment.
and enhanced MLN4924 toxicity (Figures 6a and b). The opposite effect was observed upon RPL11 knockdown, which reduced both the MLN4924-induced accumulation of cells in S-phase and cytotoxicity (Figures 6a and b). However, the effects of RPL11 knockdown on cell cycle and survival are p53-independent, as simultaneous knockdown of RPL11 and p53 did not alter the effect of RPL11 knockdown upon MLN4924 treatment (Figures 6a and b). Similar results on cell survival were obtained when instead of MLN4924, siRNAs against the catalytic subunit of NAE (ubaa3) were used (Figure 6c).

Thus, our data suggest that although the RPL11-mediated p53 activation by MLN4924 is cytoprotective, loss of RPL11 protects cells against MLN4924 by p53-independent mechanisms.

**DISCUSSION**

The NEDD8 conjugation pathway controls key cellular processes such as cell viability and cell cycle progression. The promising preclinical studies for inhibitors of NEDDylation have emphasized the importance of the NEDD8 pathway as a novel drug target. Therefore, elucidation of pathways that are controlled by NEDDylation is critical for our understanding of the mechanisms of action for these potential new chemotherapeutics. The MLN4924-induced activation of p53 is relevant to the cellular response to MLN4924 treatment, albeit the mechanism remains elusive. In this study, we show that MLN4924 activates the p53 tumour suppressor through the RP-Mdm2 pathway with characteristics of nucleolar stress. One of the pleiotropic effects of MLN4924 is to alter the morphology and to increase the size of the nucleolus. Similar effects on nucleolar size were observed in germline cells of MLN4924-treated *C. elegans*, which suggests a highly conserved role for protein NEDDylation to control the function of the nucleolus.

Changes in nucleolar size and morphology are associated with changes in rates of proliferation. Indeed, nucleolar hypertrophy is linked to malignancy, as increase in Pol I activity and ribosome production is required to sustain higher rates of growth.\(^\text{51}\) Reduction in nucleolar size or fragmentation is linked with the action of chemotherapeutics such as ActD, which inhibit Pol I function and cause cell cycle arrest.\(^\text{24,28}\) In the contrary, we found that MLN4924, a compound with anti-proliferative effects, increases nucleolar size indicating that nucleolar hypertrophy may not always be associated to higher rates of cell growth.

Quantitative proteomics showed that the MLN4924-induced morphological changes in the nucleolus are associated with a reorganization of the nucleolar proteome that are of similar scale to the well-described nucleolar ‘stressor’ ActD. However, a key difference compared with ActD, is that MLN4924 does not affect rRNA production/processing. Interestingly, MLN4924 increases the nucleolar abundance of multiple members of the MCM family of DNA replication licensing factors, along with histone acetyltransferase complexes, which are essential for DNA replication licensing\(^\text{52,53}\) (Supplementary Information S3). Indeed, we found that the replication factor MCM2 is relocalized to the peri-nucleolar area upon MLN4924 treatment (Supplementary Information S5). Therefore, the increase in nucleolar size by MLN4924 may be associated to the increase in the abundance of replication\(^\text{54}\) and acetylation factors either within the nucleolus or as nucleolar-associated proteins.

Although the effects of MLN4924 and ActD on nucleolar function are different, the downstream signalling events that lead to p53 activation are quite similar. Both compounds inhibit RPL11-NEDDylation and reduce the mobility of RPL11 in the nucleolus. Furthermore, the p53 activation induced by either compound depends on the interaction of RPL11/RPL5 with Mdm2, which is regarded as a key step in p53 activation specifically upon nucleolar stress and not upon DNA damage.\(^\text{55}\) Therefore, despite the pleiotropic effects induced because of inhibition of CRL function, p53 activation by MLN4924 depends strictly on the RP-Mdm2 module. We propose that MLN4924 causes ‘nucleolar stress’ by affecting the nucleolar proteome rather than inhibiting nucleolar transcription. The downstream biological outcome is the signalling to cell cycle regulators such as p53 that depends on the RP-Mdm2 pathway. Interestingly, combination of MLN4924 with ActD provides an additive effect on p53 stabilization, indicating that these compounds act on the same signalling pathway (Supplementary Information S6). This further supports the notion that MLN4924 activates p53 through nucleolar signalling, most likely downstream of Pol I activity regulation and upstream of the RP-Mdm2 module. Although our studies suggest that NEDDylation of RPL11 is a target of MLN4924, we cannot exclude the possibility that the observed changes in the nucleolar proteome and resulting p53 activation is indirectly due to CRL inhibition.

The nucleolus is viewed as a major hub for the detection of cellular stress, which elicits signalling events that control the activity of tumour suppressors and oncogenes.\(^\text{24}\) The plasticity and highly dynamic nature of the nucleolar proteome is a major determinant of decoding a stress signal to a signalling output.\(^\text{23}\) The changes in the nucleolar proteome upon stress conditions are directly linked to the regulation of key components of the cell cycle.\(^\text{24,29}\) To our knowledge, MLN4924 is the first example of a small compound that re-organizes the nucleolar proteome and activates p53 with all the characteristics of ‘nucleolar stress’ in the absence of inhibition of nucleolar transcription. The data suggest that targeting the nucleolar proteome may elicit the required signalling events to cell cycle regulators without affecting rRNA production and ribosome biosynthesis. This may be important as targeting Pol I activity is emerging as an attractive anti-cancer approach.\(^\text{35–37}\)

Intriguingly, neither MLN4924 nor ActD caused transcriptional activation of CEP-1/p53 in *C. elegans* despite their clear effects on nucleolar morphology and size (Figures 3a, c and 5e). Previous studies in *C elegans* showed that depletion of the nucleolar RNA-associated protein NOL-6 induces immunity against bacteria infection through activation of CEP-1/p53, suggesting that the nucleolus can signal to CEP-1/p53 under stress conditions.\(^\text{58}\) A homologue for Mdm2 has not been found in nematodes, so it is possible that MLN4924- and ActD-induced p53 activation depends strictly on the Mdm2 presence, whereas NOL-6-induced signalling depends on other E3-ligases or other mechanisms of CEP-1/p53 activity regulation (translational).\(^\text{59}\)

Although p53 activation by MLN4924 depends on RPL11, cell cycle analysis and survival assays show a differential role for p53 and RPL11 in the MLN4924 response. Our data are consistent with previous studies showing that the MLN4924-induced activation of p53 is cytoprotective.\(^\text{13}\) This is most likely due to the p53-induced G1 arrest that prevents entry of cells in the S-phase where MLN4924 elicits toxicity. In contrary, RPL11 is required for the MLN4924-induced accumulation of cells in S-phase and cytotoxicity. However, these effects are p53-independent, indicating that additional pathways controlled by RPL11 are involved in the MLN4924 biological response. For example, RPL11 controls the transcriptional activity of c-myc and cyclin expression.\(^\text{60–62}\) Indeed, the loss of RPL11 was shown to suppress cell proliferation, in the absence of cell cycle arrest, independently of p53.\(^\text{62}\) The data also indicate that the rates of cell proliferation may be a critical determinant for the effectiveness of MLN4924 as potential chemotherapeutic.

In summary, the present study shows that MLN4924 activates p53 through the RP-Mdm2 pathway and suggests that targeting the nucleolus is, at least in part, a mechanism of action for inhibitors of NEDDylation.
MATERIALS AND METHODS

Gene expression
RNA from MCF7 cells transfected with siRNAs and treated with ActD or MLN4924 was isolated and used for cDNA synthesis. The cDNA was then used for quantitative real-time PCR to monitor mdm2 expression. Primers for mdm2 (P2) promoter were previously described.63 Full details can be found in Supplementary Information.

Isolation of His6-NEDDylated proteins, ribosome profiling
Ni2+-pull downs and total cell extracts were prepared as described in the studies by Xirodimas et al.64 and Tatham et al.65 Ribosome profiling was performed as described by Xirodimas et al.36

Detection of newly synthesized RNA and rRNA processing
Transcription efficiency was monitored by 3H-uridine incorporation of newly synthesized RNAs as described.66 Briefly, H1299 cells were treated with ActD or MLN4924 and pulsed with 3H-uridine for 1 h at 37 °C. RNA was isolated and subjected to agarose-formaldehyde gel electrophoresis. For monitoring rRNA processing, H1299 cells were treated as above for 8 or 15 h and isolated total RNA was used for northern blotting with 32P-labelled probes to reveal either all 18S precursors (5′ITS1) or 28S and 5.8S (ITS2). Full details can be found in Supplementary Information.

Microscopy
Fluorescence recovery after photobleaching experiments were performed in H1299 cells stably expressing RPL11-EGFP as described by Barak et al.35

Figure 5. (a) Strategy for SILAC labelling in U2OS cells and proteomic analysis of nucleoli. (b) Profile of nucleolar proteins affected by MLN4924 or ActD. (c) Bioinformatic analysis using Panther software illustrating biological processes affected by MLN4924 and ActD. (d) Proteins affected both by MLN4924 and ActD. (e) H1299 cells were pre-treated with DMSO (5 h), ActD (5 μM) or MLN4924 (1 μM) as indicated in duplicates, and one set of cells was used for labelling for 1 h with 3H-uridine and monitoring of rRNA production. EtBr staining was used to monitor loading of total RNA. The other set of cells was used for lysis in 2× SDS lysis buffer and western blot analysis (low panel). (f) H1299 cells were treated as above for 8 or 15 h. rRNA processing was monitored with the indicated probes as described in methods. (g) H1299 cells were treated as above for 15 h. Cells were harvested and extracts were used for ribosome profiling as described in methods.

Details are included in Supplementary Information. For immunostaining in U2OS cells, samples were prepared and methods were used as described by Liu and Xirodimas. All primary antibodies were used at 1:100 dilution.

Flow cytometry
MCF7 cells in 6-well plates were transfected with 5 nM control, RPL11 and p53 siRNAs as indicated. The total amount of siRNAs was normalized to 10 nM with control siRNA. Forty-eight hours post transfection, cells were treated with either DMSO or with MLN4924 (0.5 μM) for 24 h. Cells were collected by trypsin, fixed with ethanol at 4 °C and labelled with propidium iodide for 30 min. DNA content of 10 000 cells per condition was quantified on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) using Cell Quest acquisition software and employing pulse-width analysis to exclude doublets. Data were analysed using Flowjo software (Treestar Inc., Ashland, OR, USA) and the Watson (pragmatic) model to determine cell cycle distribution.

Treatments
For each experiment of drug assay, 500 worms were treated at L4 larval stage in liquid culture of M9 medium complemented with cholesterol (5 μg/ml) and HT115 bacteria (4 × 10⁵). MLN4924 was used at 100 μM, ActD at 100 nM and doxorubicin at 100 μM. Compounds were diluted in M9 solution before use. For UV response, L4 worms were exposed at 100 J/m².
Figure 6. MCF7 cells were transfected with either control, RPL11 or p53 siRNAs as indicated. Forty-eight hours post transfection, cells were treated with DMSO or MLN4924 (0.5 μM) for 24 h before cell cycle analysis. Histograms show the cell cycle profiles and % of cell populations in different phases. (b) MCF7 cells were transfected with the indicated siRNAs and 24h later, cells were exposed to MLN4924 (1 μM) for 24, 48 or 72 h. For each siRNA experiment, survival was normalized against the control-treated (DMSO) cells, represented as 100%. (c) To mimic the experiment performed in b using MLN4924, siRNA against the catalytic subunit of NAE was used in combination with RPL11 and p53 siRNAs as indicated. Cell survival was measured 72 h later and values are presented as percentage changes over the control siRNA-transfected cells. For experiments in b and c, data presented the average of three independent experiments ± s.d.
and dissected 5 h later. After treatment of MLN4924 or Ac2D, worms were dissected or put in trizol for quantitative real-time PCR.

Nucleoli volume quantification with Image J software
Observations were performed using Zeiss (Oberkochen, Germany) Axioimager Z2. For each germline, pachytene stage was observed with ×63 objective and stacks of all the thickness were obtained (0.5 μm per stack) to see overall nucleus with Metamorph software. The same stack representing the nucleus (DAPI staining) and the nucleolus (Fibrillarin staining) was selected for further analysis. An average of 50 nuclei in human cells and 6 germlines-50 nuclei in C. elegans were used for each experiment. Full details can be found in Supplementary Information.

Subcellular fractionation and proteomics
DMEM media depleted of arginine (R) and lysine (K) were supplemented with 10% dialysed foetal calf serum and either light (K80R0) medium (K4R6) or heavy (K8R10) labelled amino acids. U2OS cells were grown in different media and after six passages full incorporation was confirmed. For each condition, 20 × 15 cm dishes were grown till 80% confluence before treatment. Subcellular fractionation and isolation of nucleoli was performed as described by Andersen et al, and Supplementary Information.

Survival assays
MCF7 cells were seeded and transfected with 5 nM of each of the siRNA pool in 24-well plates (15 000 cells per well). The total amount of siRNAs measured with the CellTiter-Glo Luminescence assay from Promega supported by ATIP/AVENIR and Marie Curie Career Integration (FP7) grants.

CONFLICT OF INTEREST
PS was an employee of Millennium Pharmaceuticals at the start of the studies. The other authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We are grateful to Dr Yanping Zhang for providing the wild-type and mutant Mdm2 C305F MEFs, the Montpellier RIO Imaging facility and Stefano Fumagalli for critical reading of the manuscript. AB and AP are supported by an ANR (National Agency for Research) postdoctoral fellowship awarded to AB. The project in DPX laboratory was supported by ATIP/AVENIR and Marie Curie Career Integration (FP7) grants.

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Oncogene (2015) 1 – 12
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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)