Abstract

Origin licensing is a process responsible for preparing the genome for replication during the cell cycle. The overexpression of chromatin licensing and DNA replication factor 1 (Cdt1), an origin licensing protein, is toxic to cells because it induces re-replication of DNA. Protein activity is often regulated through the mechanism of phosphorylation. However, the phospho-regulation of Cdt1 during the cell cycle is not fully understood. This study investigates how phosphorylation of two newly identified phosphorylation sites (S93 and T152) regulates Cdt1 function during origin licensing. We generated three human U2OS cell lines harboring doxycycline inducible mutationally altered versions of Cdt1 [S93A, T152A, and a combination of the two ("2A")] via standard cloning methods. We performed colony forming assays by overexpressing ectopic Cdt1 to determine the cellular phenotypes of each mutant cell line. Non-phosphorylatable Cdt1 proteins, especially 2A, cause reduced cellular toxicity compared to WT, indicating hypomorphic activity. DNA damage markers, such as phospho-Chk1, are highly activated in cells with overexpressed Cdt1 WT. While we observed low activation of phospho-Chk1 in cells with overexpressed phosphomutants, further tests are needed to distinguish the extent of phospho-Chk1 activation between S93A and 2A. Overexpressed Cdt1 WT induces the highest incidence of re-replication, followed by Cdt1 S93A, and Cdt1 2A, as visualized by flow cytometry. These results suggest phosphorylation at S93 and T152 both contribute to Cdt1 function in origin licensing. Future studies of how phosphorylation of Cdt1 residues S93 and T152 affects Cdt1 protein dynamics and cell growth will lead to a better understanding of the control mechanisms of Cdt1.

Introduction

All cells have the complex task of copying their entire contents including their genome and organelles during the cell cycle. The end result of this process is the creation of two identical daughter cells. This coordinated cycle must be precisely controlled to ensure each individual event proceeds correctly; otherwise, DNA damage or abnormally segregated chromosomes can be passed on to daughter cells, which may lead to the uncontrolled proliferation of cells and cancer. One protein necessary for the progression of the cell cycle is chromatin licensing and DNA replication factor 1 (Cdt1).

First, Cdt1 is required for origin licensing, which prepares the DNA for replication during G1 phase. This process involves the assembly of protein components to form the pre-replication complex (Cook, 2009). During origin licensing, Cdt1 with the help of cell division cycle 6 (Cdc6) and origin recognition complex (ORC), is responsible for loading the hexameric minichromosome maintenance protein (MCM)2-7 helicase onto DNA origins to prepare them for replication (Cook, 2009). *In vitro*, Cdt1 binds with MCM4/6/7 complex and stimulates MCM DNA helicase activity (You & Masai, 2008). While *in vivo*, human Cdt1 C-terminus forms a complex with MCM6 C-terminus to promote MCM loading (Wei et al., 2010). Recently, another role of Cdt1 has been elucidated. Cdt1 is essential for kinetochore-microtubule attachment during metaphase of the cell cycle, facilitating the segregation of chromosomes. Cdt1 associates with kinetochores through the interaction of Hec1 component of the Ndc80 complex (Varma et al., 2012).

If Cdt1 is active during inappropriate times, such as in S phase, Cdt1 induces DNA replication multiple times instead of once, creating a toxic event (Vaziri et al., 2003). The consequences of re-replication include cell damage, cell death and genome instability. One way

to cause inappropriate activity and subsequent toxicity is to overexpress Cdt1, as will be utilized in the assays presented in this paper. Thus, if its activity is not controlled, Cdt1 can act during inappropriate stages of the cell cycle and cause DNA damage, leading to cell death or cancer formation (Vaziri et al., 2003). Likewise, mutational alterations of amino acids in Cdt1 have been found in human cancers, such as colon and lung cancer, and in a type of primordial dwarfism called Meier-Gorlin Syndrome (Liontos et al., 2007; Human Protein Atlas).

Given the functional importance of Cdt1 in the cell cycle, it is crucial that Cdt1 activity is regulated during precise stages of the cell cycle. One mechanism by which proteins are controlled is phosphorylation, the addition of a phosphate group onto the protein. Phosphorylated proteins can be subsequently activated or inactivated, which can affect the protein's ability to catalyze reactions. Additionally, phosphorylation can affect the protein's ability to bind with other proteins or can affect the protein's degradation (Cheng, Qi, Paudel, & Zhu, 2011; Johnson & Barford, 1993; Suryadinata, Sadowski, & Sarcevic, 2010). After the cell goes through the G1/S checkpoint, to ensure the genome is prepared for replication, the cell transitions into S or "synthesis" phase where the genome is replicated. During this transition, Cdt1 is degraded by the proteasome due to multiple ubiquitin-mediated proteolytic mechanisms and inactivated through geminin binding (Nishitani, Lygerou, & Nishimoto, 2004). For instance, cyclin A-Cdk2 phosphorylates T29 at the N-terminus of Cdt1. After phosphorylation, SCF-Skp2 E3 ligase binds to Cdt1 and causes subsequent ubiquitination and degradation of Cdt1 (Nishitani et al., 2006; Sugimoto et al., 2004).

Many phosphorylation sites have been identified on Cdt1, yet it is still unknown how the activity of Cdt1 is precisely regulated through phosphorylation. Apart from several phosphorylation sites that have been previously identified to affect Cdt1's function and stability,

mass spectrometry has identified two new phosphorylation sites at amino acids 152 and 93 (Hornbeck et al., 2012; Mertins et al., 2013; Sharma et al., 2014). These sites are potentially phosphorylated by cyclin A-dependent kinases (Cdks) or mitogen-activated protein kinases (MAPKs) since S93 and T152 are followed by prolines (Chandrasekaran, Tan, Hall, & Cook, 2011; Nishitani et al., 2006). The focus of this study is to explore how these two phosphorylation sites affect Cdt1's function in replication and mitosis. To accomplish this goal, we will examine the cellular phenotypes of human osteosarcoma (U2OS) cell lines containing mutationally altered versions of Cdt1. Furthermore, to assess the activity of these mutationally-altered Cdt1 proteins, we will examine DNA damage and re-replication through the expression of DNA damage markers and flow cytometry. Overall, understanding how phosphorylation impacts Cdt1's function will help us better understand Cdt1's role in the cell cycle and how it contributes to human disease.

Materials & Methods

Plasmid Preparation

For expression in mammalian cell lines, gene sequences for wild-type Cdt1, S93A Cdt1, T152A Cdt1, and 2A Cdt1, all bearing an HA epitope tag, were amplified through PCR. cDNAs coding for these proteins were subcloned into pENTR vectors. Furthermore, DNA sequencing via UNC sequencing facilities (UNC-CH, NC) of the four pENTR vectors confirmed mutations in the respective amino acids. pENTR vectors were subsequently transferred into pDESTJC16 expression vectors through recombinational Gateway® cloning (LR clonase, Invitrogen) and into DH5α *E.coli* for transformation. Bacteria were plated onto ampicillin plates and incubated overnight. Subsequently, pDEST vectors were purified from selected colonies using standard accepted boiling miniprep protocol. After restriction digest, vectors were electrophoresed on a

1% agarose gel stained with ethidium bromide to determine which colonies were positive for expression vectors. Colonies with Cdt1 S93A vector were analyzed with SpH*I* restriction enzyme. Colonies with Cdt1 WT, T152A and 2A vectors were analyzed with Afe*I* restriction enzyme. Lastly, expression vectors were purified through a midiprep and column purification (GenElute HP Plasmid Midiprep Kit, Sigma).

Cell Culture and Stable Cell Line Generation

All cell lines were grown in standard Dulbecco modified Eagle medium (DMEM, Sigma) with 10% fetal bovine serum (FBS) and 1000X penicillin/streptomycin. To generate stable cell lines, Flp-FRT_U2OSa_tetON human osteosarcoma mammalian (U2OS) cells were plated at a concentration of $3.0*10^5$ cells/well 1 day before transfection. Expression vectors were transfected into U2OS cells using the DNA transfection agent X-tremeGENE 9 (9:1 ratio of flippase to FRT-pDEST expression vectors or BS-U6GFP for control). U2OS cells, derived and provided by the Aster Lab, utilize a Flp-In™ System (Thermo Scientific) which enables the expression vectors to become site-specifically integrated within the genome of the U2OS cells via Flp recombinase-mediated DNA recombination at the FRT site (Malecki et al., 2006; O'Gorman, Fox, & Wahl, 1991). Furthermore, using this system enables us to generate stable cell lines that have inducible expression (Thermo Scientific). This cell line specifically has a doxycycline inducible expression system. Additionally, puromycin was utilized to select for cells positive for the expression vector (which contains a puro^R gene). Once puro selection was complete, the expression of exogenous and endogenous Cdt1 in these mutant Cdt1 cell lines was confirmed by inducing ectopic expression of the mutationally altered versions of Cdt1 with either full doxycycline (1 µg/ml) or a serial dilution of doxycycline (0, 0.01, 0.05, 0.1, 1 µg/mL). Ectopic expression was confirmed through immunoblot analysis.

Protein Lysate Preparation

Whole-cell lysates were prepared by first washing cells with 1X phosphate buffered saline (PBS) and trypsinizing into 500 μL of trypsin. Subsequently, cells were harvested using 10% fetal bovine serum (FBS) in 1X PBS and spun at 2,000 rpm for 2 minutes. After removing the supernatant, cells were washed in 1X PBS and spun at 2,000 rpm for 2 minutes. To store before lysing, cells were snap-frozen using dry ice and tubes were stored in -80 degrees. After harvesting, equal cell numbers were lysed in CSK buffer supplemented by 0.5% Triton X-100 detergent, protease and phosphatase inhibitors, and ATP (Cook et al., 2002). Bradford assays were performed to determine protein concentration of each extract to standardize protein concentration for immunoblot analysis. Using different bovine serum albumin (BSA) concentrations, a standard curve was created. Extract concentrations were interpolated from the standard curve. Afterward, 2X Laemmli sample buffer (SDS) with 10% β-mercaptoethanol (BME) was added to lysed cells in preparation for immunoblot analysis.

Immunoblot

First, lysates were boiled for 5 minutes and centrifuged to shear the DNA. Lysates and 2.5 μL of Precision Plus Protein™ Dual Color Standards ladder (Bio-Rad) were loaded on a 10% SDS-PAGE gel and electrophoresed at 170 V for 1 to 1.5 hours. To evaluate the expression of Cdt1 and HA ~10 μg of protein were loaded whereas ~20 μg of protein were loaded to evaluate the expression of pChk1 and pKAP1. Proteins were transferred onto either a Polyvinylidene Fluoride (PVDF) (Thermo Scientific) or nitrocellulose membrane (GE Healthcare Life Sciences) at either 110 V for ~1.5 hours or 20 V overnight. PVDF membrane was used when probing for Cdt1, HA, and tubulin and a nitrocellulose membrane was used when probing for pChk1 and

pKAP1. To confirm uniform protein loading, membranes were stained with Ponceau S solution (Sigma) before blocking.

Blots were blocked in either 5% BSA in 1X Tris buffered saline with Tween® (TBST) (for Cdt1 blots) or 5% nonfat dry milk in TBST (for HA, tubulin, pChk1, and pKAP1) for 1 hour or overnight at 4° C. Additionally, pChk1 and pKAP1 blots were washed once with 1X TBST after blocking to remove excess milk residue. For primary antibodies, membranes were incubated at either 1:10,000 anti-rabbit Cdt1 D10F11 (Cell Signaling Technology) in 5% BSA-TBST, 1:2,000 anti-rabbit phospho KAP-1 (S824) in 1X TBST (Bethyl), 1:3,000 anti-rabbit phospho-Chk1 (Ser345) in 1X TBST, or 1:50,000 anti-mouse α-Tubulin (Sigma, #9026) in 2.5% milk-TBST for 1 hour to detect for the presence of Cdt1, pKAP1, pChk1, and tubulin, respectively. Additionally, HA tag was probed, which is fused to the C-terminus of the Cdt1 expression vector, to ensure that the band present on the immunoblot was exogenous Cdt1. To evaluate the expression of HA, blots were incubated at 1:1,000 anti-rabbit HA (Bethyl) in 5% milk and 5% donkey serum in TBST. Recent blots were probed at 1:3,000 anti-rat high affinity HA (Roche) in 2.5% milk-TBST.

After incubating in primary antibody, membranes were washed in 1X TBST 3 times for 5 minutes before secondary antibody. Donkey anti-rabbit HRP secondary antibody (Jackson ImmunoResearch Laboratories) at 1:10,000 in 1.25% milk-TBST or 5% BSA-TBST, goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch Laboratories) at 1:10,000 in 1.25 % milk-TBST, or donkey anti-rat HRP secondary antibody (Jackson ImmunoResearch Laboratories) at 1:10,000 in 1.25% milk was added for 1 hour. All blots were washed in 1X TBST 3 times for 5 minutes before visualizing protein expression with Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, UK). Transferring or blocking

overnight did not affect visualization of protein expression, thus depending on time constraints

we transferred or blocked at either the time period previously stated or overnight.

Probed Protein	Blocking	Primary Antibody	Secondary Antibody
Cdt1	5% BSA-TBST	1:10,000 anti-rabbit Cdt1 in 5% BSA-TBST	1:10,000 donkey anti-rabbit HRP secondary antibody in 5% BSA-TBST
HA	5% milk-TBST	1:1,000 anti-rabbit HA (Bethyl) in 5% milk and 5% donkey serum in TBST 1:3,000 anti-rat high affinity HA (Roche) in 2.5% milk-TBST	1:10,000 donkey anti-rabbit HRP secondary antibody in 1.25% milk-TBST 1:10,000 donkey anti-rat HRP secondary antibody in 1.25% milk
pChk1	5% milk-TBST	1:3,000 anti-rabbit phospho-Chk1 (Ser345) in 1X TBST	1:10,000 donkey anti-rabbit HRP secondary antibody in 1.25% milk-TBST
pKAP1	5% milk-TBST	1:2,000 anti-rabbit phospho KAP-1 (S824) in 1X TBST	1:10,000 donkey anti-rabbit HRP secondary antibody in 1.25% milk-TBST
Tubulin	5% milk-TBST	1:50,000 anti-mouse α-Tubulin (Sigma, #9026) in 2.5% milk-TBST	1:10,000 goat anti-mouse HRP secondary antibody in 1.25 % milk-TBST

Table 1. Immunoblot conditions used.

Re-Replication Associated DNA Damage Marker Expression

To evaluate the expression of re-replication associated DNA damage markers, doxycycline inducible WT Cdt1, Cdt1 S93A, Cdt1 T152A, and Cdt1 2A U2OS cell lines were plated at a concentration of $1*10^5$ cell/mL. While plating, doxycycline (1 μg/ml) was added to the plates. As a positive control, 2 mM hydroxyurea (HU) and 0.3 μM of the Nedd8-activating enzyme inhibitor MLN4924 were added to control cells 24 hours before harvesting. Furthermore, GFP cells were UV-irradiated (20 J/m^2) 1 hour before harvesting. Cells were grown for a time

course of 24 hours, 42 hours, or 72 hours with doxycycline. Cells were harvested and protein concentration was quantified using the above protein lysate protocol.

Colony Forming Assay

U2OS cells expressing ectopic Cdt1 (WT, S93A, T152A, and 2A) and GFP, as a control, were plated at a density of 500 cells/plate in 6 cm plates and $3.0*10^5$ cells/well for immunoblot analysis. Additionally, either serial doxycycline doses (0.02, 0.04, 0.08. and 0.1 µg/mL) were added to the cells or only one doxycycline concentration (1 µg/ml) was added. For immunoblot analysis, negative control GFP cell lines were also plated. Doxycycline (1 µg/ml) was added during plating, 0.3 µM MLN4924 was added 24 hours before harvesting, and UV (20 J/m^2) was applied 1 hour before harvesting. As for cell culture, 20% FBS in DMEM was used for the colony forming cells and 10% FBS in DMEM was used for immunoblots analysis. Cells for the immunoblots analysis were harvested 24 hours or 3 days after plating using the above protein lysate protocol. Whole cell lysates were prepared using the standard procedure with CSK buffer as mentioned above. Colonies for the colony forming assay were stained with crystal violet 10 days after plating. Pictures of the plates were taken using a Canon 900F Mark II scanner. From these images, colony count and average colony size were quantified using ImageJ software. Data was analyzed using Microsoft Excel 2010.

Flow Cytometry

U2OS cells expressing ectopic Cdt1 (wild type, S93A, T152A, and 2A) were plated at a concentration of $1.5*10^5$ cells/mL with 1 µg/mL doxycycline added. Additionally, the U2OS cell line with ectopic GFP was plated with and without 0.3 µM of MLN4924 as a control. After three days, cells were labeled with 10 µM 5-ethynyl-2'-deoxyuridine (EdU) for 30-45 minutes. Next, cells were trypsinized and harvested using 10% FBS in PBS. Subsequently, cells were pelleted at

1,000xg for 4 min and resuspended in PBS both times. Approximately half of the volume was saved for immunoblot analysis and the remaining cells were pelleted. Afterward, cells were fixed for flow cytometry analysis with 4% paraformaldehyde (PFA) and 0.5 mL of PBS for 15 minutes at room temperature. After fixing, cells were pelleted and resuspended two times and stored in 4°C until staining. To prepare cells for staining, cells were pelleted, resuspended and incubated in PBS with 1% BSA for 5 minutes. Next, cells were pelleted, resuspended and incubated in PBS + 1% BSA + 0.5% Triton X-100 for 15 minutes. After pelleting, cells were resuspended in labeling solution consisting of 1 mM CuSO4, PBS, 0.2-2 µM Alexa Fluor 647 Azide (Jackson ImmunoResearch Laboratories), and 100 mM ascorbic acid and incubated for 30 minutes in a dark area. After pelleting, cells were resuspended with 1% BSA-0.5% Triton X-100-PBS. Cells were pelleted a final time at 1,000xg for 4 minutes and resuspended in 1% BSA with 0.5% Triton X-100 in PBS supplemented with 100 µg/mL RNAse and 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) stain. Cells were vortexed and stored in 4°C overnight, protected from light, to stain all DNA. Flow cytometry analysis was performed using a cyan FACScan (DakoCytomation) at UNC Flow Cytometry Core Facilities (UNC-CH, NC) and Summit version 4.4 software.

Results

Creation of Stable Mutant Cdt1 Cell Lines and Ectopic Expression of Mutationally-Altered Cdt1 Proteins

The purpose of this study is to investigate the phosphorylation sites at amino acids 93 and 152, and whether these sites affect Cdt1 function in origin licensing and mitosis. We first created cell lines with exogenous Cdt1 vectors containing mutational alterations at either amino acid 93, 152, or both amino acids (2A). The diagram in Figure 1A shows where these two

phosphorylation sites fall within the Cdt1 vector we created. It is important to note that amino acids 93 and 152 fall within the N-terminus near the Hec1 binding domain. Thus, mutating these phosphorylation sites may affect Cdt1 binding to proteins such as Hec1 component of Ndc80 complex. This expression vector also has an HA epitope tag derived from the hemagglutinin molecule (amino acids 98-106) attached to the Cdt1 protein sequence (Abcam). Having an HA-tagged protein enables us to probe for HA and specifically observe ectopic Cdt1, rather than endogenous Cdt1, in experiments. Normally, the amino acids serine and threonine can be phosphorylated and have a highly negative charge. We decided to replace amino acids at S93 and T152 with nonpolar alanines, whereby these sites would be unable to be phosphorylated. Thus, we are interested in seeing if mutating these phosphorylation sites affects Cdt1 function during origin licensing and mitosis.

WT Cdt1 overexpression leads to cellular toxicity by re-licensing and re-replicating DNA, ultimately causing genome instability (Vaziri et al., 2003). Thus, we created stable cell lines with doxycycline induction systems to study the function of these two phosphorylation sites. Under a doxycycline induction system we can control when to overexpress WT Cdt1 or the phosphomutant proteins. We transfected human U20S cells with vectors for either WT Cdt1 or one of the three mutationally altered versions of Cdt1 (S93A, T152A, and 2A) and confirmed the ectopic expression by immunoblot analysis after induction (Figure 1B and 1C). In the immunoblot, the bottom band is endogenous Cdt1 while the top band is ectopic Cdt1 (WT, S93A, T152A, 2A), marked as Cdt1-HA. With an HA tag, the ectopic version of Cdt1 has a larger kDa value than endogenous Cdt1.

With an increase in doxycycline concentration, ectopic Cdt1 T152A and 2A increased in expression, as shown by a larger kDa band above endogenous Cdt1 (Figure 1B). Additionally,

maximum doxycycline concentration (1 µg/ml) induced the expression of ectopic Cdt1 WT and

S93A (Figure 1C). In all cell lines (WT, S93A, T152A, and 2A), maximum doxycycline

concentration induced ectopic Cdt1 (upper band) expression above endogenous Cdt1 (lower

band) expression. Reprobing this immunoblot with HA antibody confirmed that the top band is

indeed the ectopic version of Cdt1, which contains a HA tag (data not shown). These results

indicate that this expression vector was highly controlled and stably expressed by doxycycline.
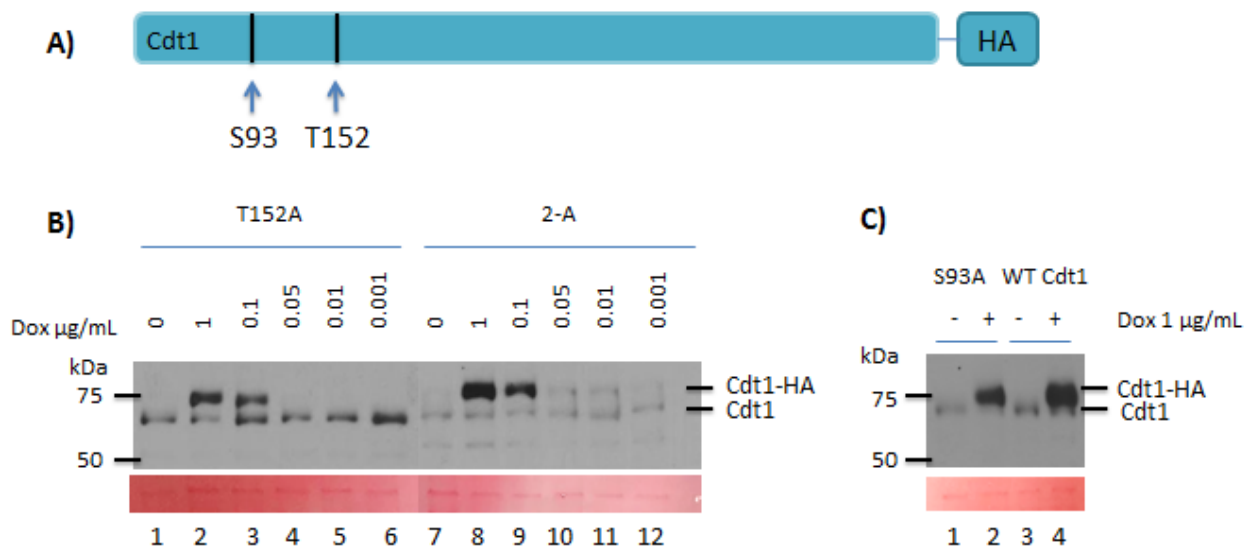
Figure 1. Doxycycline Inducible Cdt1 Expression in transfected U2OS Cells. A) Diagrammatic representation of Cdt1 vector with the two phosphorylation sites of interest, S93 and T152, and a HA tag. **B-C)** Immunoblot demonstrating stable expression of ectopic Cdt1 proteins **(B)** T152A and 2A, **(C)** WT Cdt1 and S93A in U2OSa cells, under the control of doxycycline. The lower band is endogenous Cdt1 and the upper band is ectopic Cdt1 (either WT or the mutationally-altered protein). Puromycin resistant U2OSa cells were treated with doxycycline, at the indicated concentrations, 24 hours before harvesting. Ponceau staining of the immunoblots is provided to illustrate equal loading of protein.

Establishing a Cellular Toxicity Assay with WT Cdt1 Overexpression

Given that Cdt1 overexpression leads to re-licensing and re-replication, we performed a

colony forming assay to characterize the cellular phenotype of our generated WT Cdt1 cell line.

These results would establish a baseline in which we could subsequently compare the effect of

the mutationally-altered Cdt1 proteins on cell growth. Cdt1 overexpression has been shown to reload MCM helicase during S phase, eliciting re-replication (Vaziri et al., 2003). Thus, in this experiment we hypothesized Cdt1 overexpression would lead to cellular toxicity. Increasing ectopic Cdt1 expression -through serial doxycycline concentrations- decreased cellular proliferation, as observed by reduced cell growth/colony formation in dishes (Figure 2A). Since doxycycline induces ectopic expression, the differences in colony growth are a direct result of Cdt1 overexpression. We repeated this experiment three times and used ImageJ to quantify colony counts and colony size (Figure 2B). At doxycycline concentrations of 0.08 μg/ml and 0.1 μg/ml, when ectopic expression of Cdt1 was higher than endogenous expression, colony counts were dramatically reduced in comparison to cells without doxycycline (Figure 2C). At both doxycycline concentrations, colony count was reduced by ~2 fold, in comparison to GFP cells with and without doxycycline. Thus, ectopic Cdt1 overexpression led to toxicity and reduced cellular proliferation.
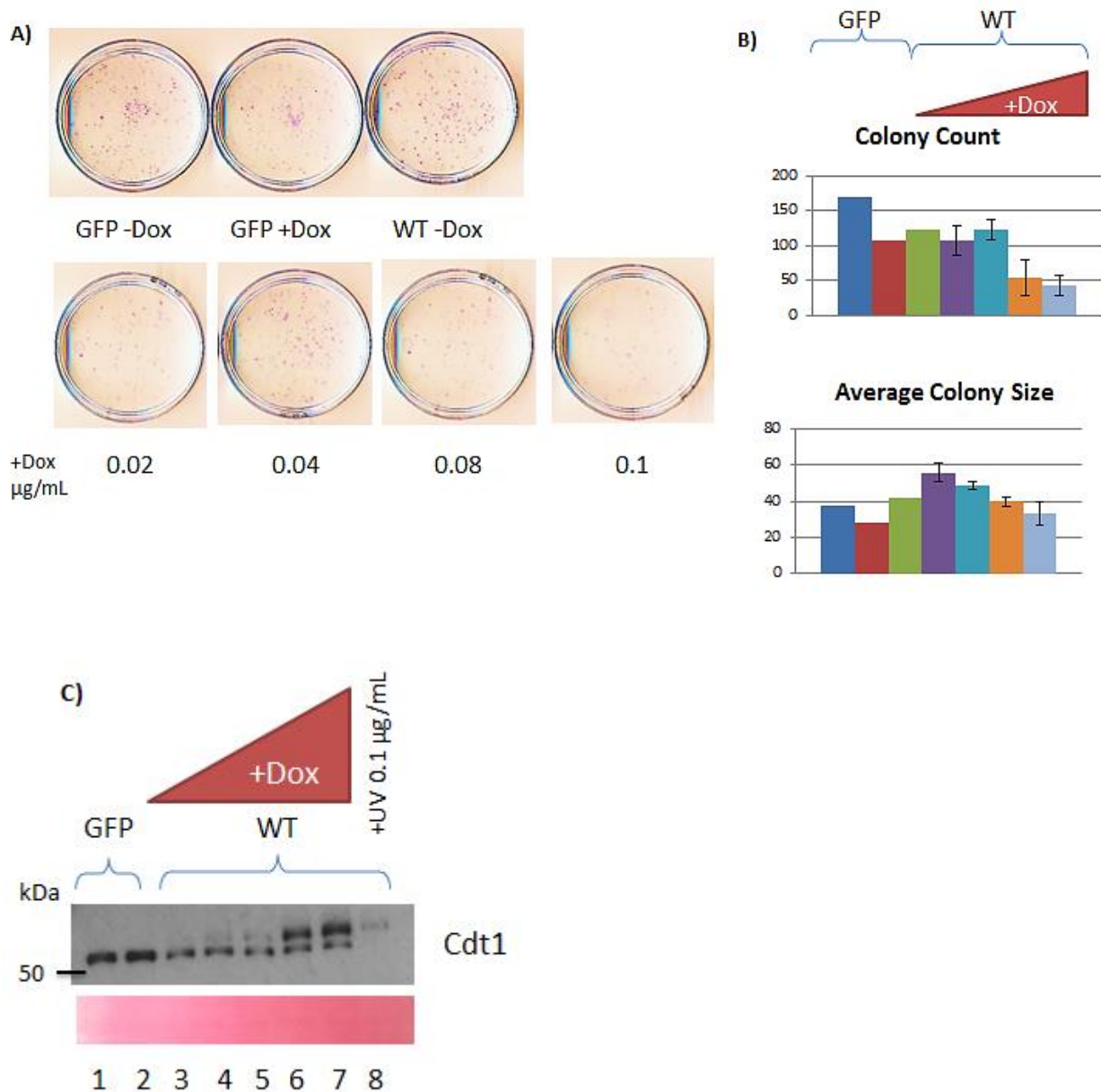
Figure 2. Colony forming assay with WT Cdt1 Overexpression. **A)** Colony forming assay illustrating cell growth of U2OS cells overexpressing either ectopic Cdt1 or GFP (control). -Dox denotes cells that did not receive doxycycline and +Dox denotes cells that receive different concentrations of doxycycline (0.02 μg/ml, 0.04 μg/ml, 0.08 μg/ml, or 0.1 μg/ml). Increasing doxycycline concentrations were used to overexpress Cdt1 at increasing levels. Cells were plated at a density of 500 cells/plate on 6 cm dishes. Colonies were stained with crystal violet after 10 days of growth. **(B)** Colony number and size were quantified using ImageJ software and graphed. Standard deviation bars were calculated from three sets of technical replicates (n=3) for each doxycycline concentration (0.02 μg/ml, 0.04 μg/ml, 0.08 μg/ml, or 0.1 μg/ml). **C)** Immunoblot illustrating ectopic expression of Cdt1 with various doxycycline concentrations. The lower band is endogenous Cdt1 and the upper band is ectopic WT Cdt1 with an HA tag. UV (20 J/m^2) was applied 1 hour before harvesting to one of the WT Cdt1 U2OS cells with 1 μg/mL of

doxycycline to check for antibody specificity to Cdt1 in the immunoblots. UV damage causes PCNA to promote the degradation of Cdt1, therefore by using this control we can ensure the antibody observed in the immunoblot is Cdt1. Ponceau staining of the immunoblot is provided to illustrate equal loading of protein.

Overexpression of Cdt1 phosphomutant proteins Exhibit Reduced Cellular Toxicity

To determine the cellular phenotype of the Cdt1 mutationally-altered proteins (S93A, T152A, and 2A), we performed colony forming assays on cells by overexpressing ectopic WT or phosphomutant forms of Cdt1. Results indicate a drastic difference in colony growth between cells with overexpressed WT Cdt1 and cells with overexpressed Cdt1 phosphomutant proteins (Figure 3A). In comparison to dishes with overexpressed WT Cdt1, cells with overexpressed Cdt1 phosphomutant proteins (S93A, T152A, and 2A) had less inhibition of colony formation. Comparing all three phosphomutant cell lines, cells with overexpressed Cdt1 T152A and 2A formed more colonies than cells with overexpressed Cdt1 S93A. Additionally, cells with overexpressed Cdt1 T152A and 2A had comparable levels of colony growth to one another. Quantifying the colonies, as seen in Figure 3B, confirms this pattern. We quantified colony formation with ImageJ and graphed the percent of colony formation in cells with doxycycline relative to the same cell lines without doxycycline induction for three biological replicates. Cells with overexpressed Cdt1 WT, S93A, T152A, and 2A formed colonies at 7.3%, 40.5%, 68.5%, and 61.7%, respectively, relative to the same cell lines without doxycycline, set to 100% colony formation. This equates to a ~5.6, ~9.4, and ~8.5 fold increase in colony formation for Cdt1 S93A, T152A, 2A, respectively, in comparison to WT Cdt1. Immunoblot analysis indicated that ectopic expression of Cdt1 WT, S93A, and 2A was above endogenous levels, while ectopic expression of Cdt1 T152A was about the same as endogenous levels (Figure 3C).

To accurately account for the differences in ectopic expression of the Cdt1 protein, we quantified the expression of ectopic Cdt1 phosphomutant proteins relative to tubulin and

normalized all values to WT Cdt1. When WT Cdt1 was set to 100% inhibition of colony formation, S93A and 2A inhibited colony formation by 77.6% and 45.9%, respectively (Figure 3D). These results indicate that Cdt1 S93A elicited slightly less toxicity than WT Cdt1, while Cdt1 2A caused dramatically less toxicity than WT Cdt1. Thus, S93A and 2A mutational alterations of Cdt1 were hypomorphic and had lower levels of activity than WT Cdt1.
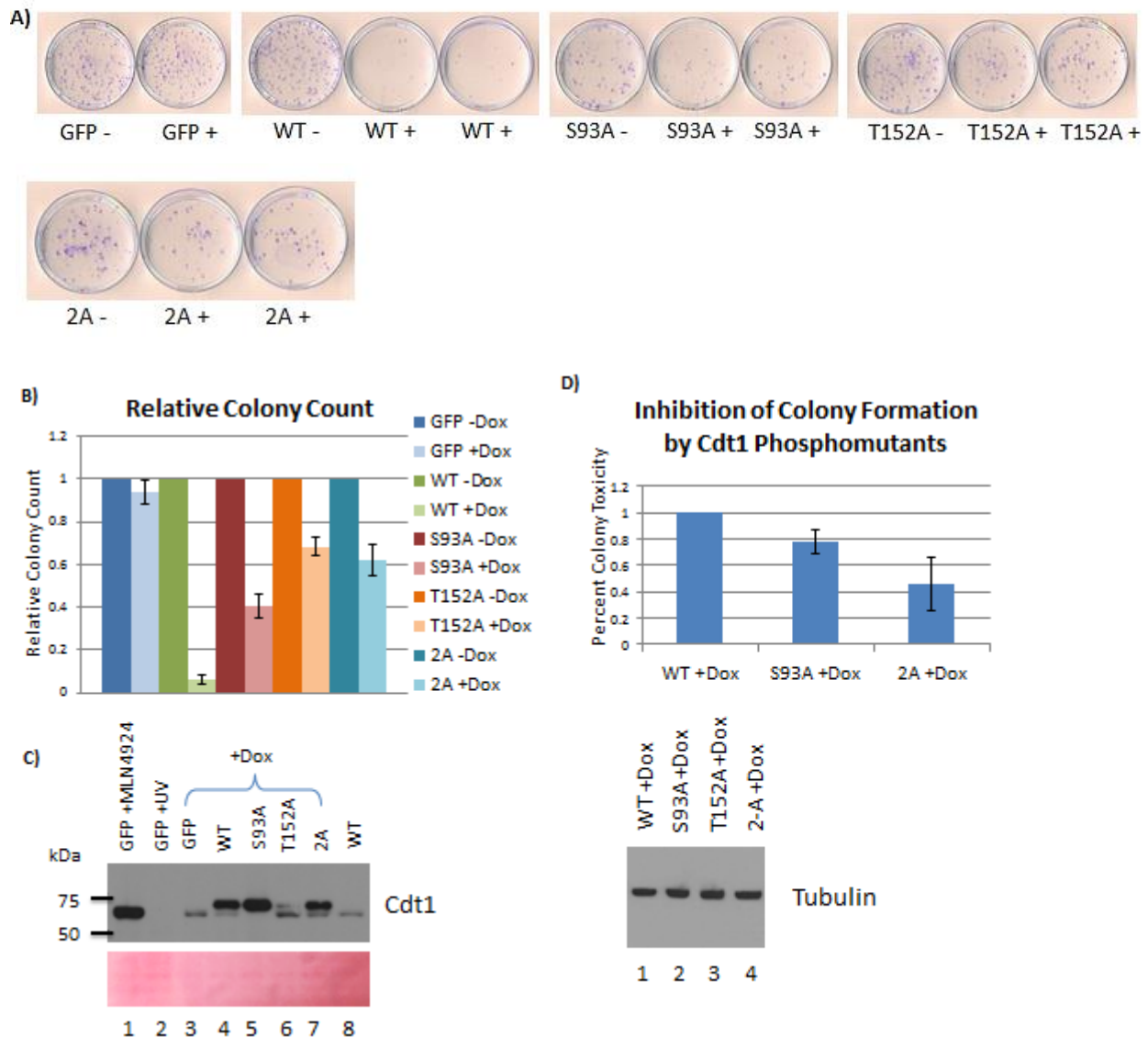

Figure 3. Colony forming assay with Cdt1 phosphomutant overexpression. A) Representative cell cultured plates (6 cm) illustrating colony growth in colony forming assay. Doxycyline (1 μg/ml) was added to all plates denoted with a "+" to induce ectopic overexpression of either GFP, WT Cdt1, or the phosphomutants. The "-" denotes no doxycycline

added. **B)** Colonies were quantified with ImageJ and standard deviations calculated from three sets of biological replicates, each comprised of two technical replicates. The induction of Cdt1 WT or Cdt1 phosphomutant protein is denoted by +Dox. Colony counts are graphed relative to the respective –Dox, which are normalized to 1. **C)** Immunoblot illustrating ectopic expression (top band) and endogenous expression (bottom band) of Cdt1. Ponceau staining of the immunoblot is provided to illustrate equal loading of protein. **D)** Ectopic expression of Cdt1 and expression of tubulin from the immunoblot was quantified with ImageJ and used to normalize cell proliferation results based on the protein expression. Representative immunoblot, shown below the graph, demonstrates tubulin expression from one of the biological replicates. Data in the graph include two sets of biological replicates.

Effect of Cdt1 Overexpression in Re-replication Associated DNA Damage

It is previously known that if Cdt1 is active outside of G1, it can cause relicensing and re-replication (Fujita, 2006; Vaziri et al., 2003). To further explore why we saw a difference in cell proliferation in colony formation and to test the possibility that Cdt1 (WT and phosphomutants) overexpression induces different levels of re-replication, we checked for re-replication associated DNA damage markers in the same doxycycline inducible WT Cdt1 and phosphomutant cell lines. We hypothesized that less inhibition of colony formation in cells with Cdt1 phosphomutant proteins is due to their decreased ability to aberrantly initiate re-replication.

To determine whether the observed decreased inhibition of colony formation from overexpression of Cdt1 phosphomutant protein was due to reduced re-replication, we examined whether DNA damage markers were activated. One DNA damage marker is Chk1, which becomes phosphorylated by the Ataxia telangiectasia and Rad3 related (ATR) DNA damage response pathway signal transduction cascade (Piwnica-Worms, 2001). We first overexpressed Cdt1 WT (through doxycycline application) to optimize experimental conditions such as antibody concentration and plating time before experimenting with the phosphomutant cell lines. Since it takes roughly 24 hours for cells to complete one cell cycle, we decided to apply doxycycline for three days, enabling cells to go through multiple cell cycles and examine the effect of presumed re-licensing (Solly, Wang, Xu, Strulovici, & Zheng, 2004). We expected to

observe high activation of phospho-Chk1 in cells with overexpressed ectopic WT Cdt1. Phospho-Chk1 levels were indeed activated when Cdt1 was overexpressed when compared to control (cells without doxycycline induction). However, ectopic expression of Cdt1 was close to endogenous levels of Cdt1 (data not shown). Next we set up a doxycycline time course to determine the optimal time for expression of both ectopic Cdt1 and activation of phospho-Chk1. We examined times ranging from 4 to 72 hours of doxycycline and examined the expression of Cdt1 and HA (which probes for ectopic Cdt1), as well as activation of phospho-Chk1. In this experiment, we observed the highest activation of pChk1 and expression of ectopic Cdt1 between 24 and 48 hours.

Using the optimized timeframe between 24 and 48 hours, we doxycycline induced ectopic Cdt1 WT and phosphomutant protein in the cell lines for 24 and 42 hours to examine the phospho-Chk1 activation levels. In the controls, lanes 1-4, only endogenous levels of Cdt1 were observed, as expected, except for negative control cells with UV. We expected to observe little to no endogenous Cdt1 expression in cells with UV due to the inhibitory effect of UV damage on pre-replication complex formation. This may be one reason why phospho-Chk1 was not activated highly in positive control cells with UV (lane 4). Regardless, expression of phospho-Chk1 due to HU can be used as a positive control, since HU causes genome instability by stalling replication forks (lane 3) (Coleman et al., 2015). In general, ectopic expression of Cdt1 remained relatively constant at 24 and 42 hours and higher than endogenous Cdt1 in lanes 5-12, excluding lanes 9 and 10 (Figure 4). Focusing on the activation of phospho-Chk1 at 24 hours, phospho-Chk1 is highly activated in cells with WT Cdt1 (lane 5), at an activation level slightly below the HU positive control (lane 3). Furthermore, phospho-Chk1 was activated at lower levels in the cells with phosphomutant proteins (lanes 7 and 11) than in cells with WT Cdt1 (lane 5). More

specifically, cells with ectopic Cdt1 S93A (lane 7) had lower levels of phospho-Chk1 activation than cells with ectopic WT Cdt1 (lane 5), but had slightly higher levels of phospho-Chk1 activation than cells with ectopic Cdt1 2A (lanes 11). Additionally, cells with ectopic Cdt1 2A (lanes 11) had low levels of phospho-Chk1 activation in comparison to WT Cdt1 (lane 5), however this may be due to low ectopic expression of Cdt1. Conclusions cannot be made about Cdt1 T152A because ectopic expression was near endogenous levels. In the future, this experiment will be repeated using the 24 hour time point to ensure replicability.

Overall, the phospho-Chk1 expression results are in line with the results from the colony forming assays. Cdt1 S93A and 2A phosphomutant proteins are less active than WT Cdt1 and activate the DNA damage marker phospho-Chk1 to a lesser extent than WT Cdt1.
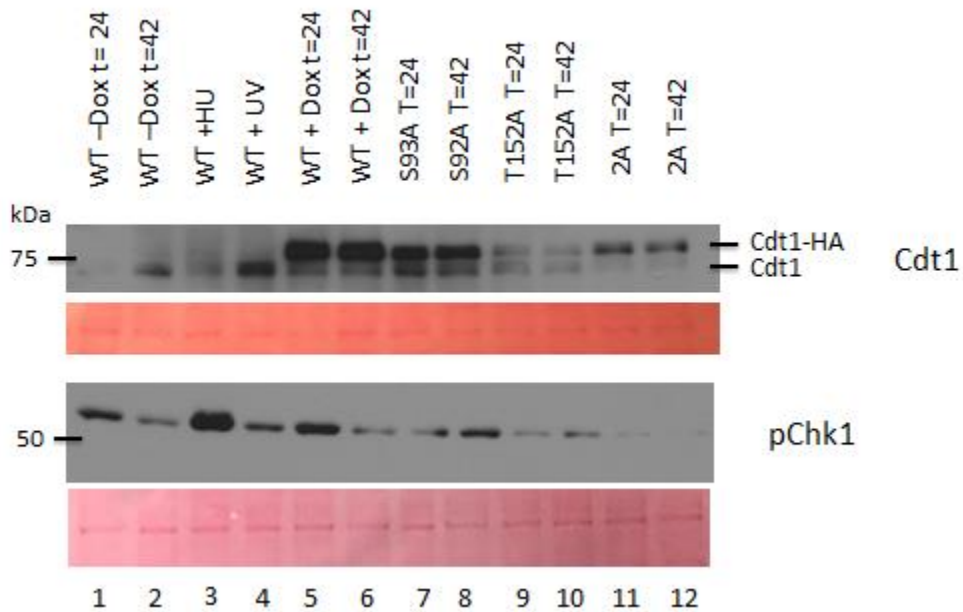

Figure 4. Activation of DNA Damage Marker (pChk1) with overexpressed Cdt1. Doxycycline (1 µg/ml) was added for 24 or 42 hours. HU and UV serve as the positive control for pChk1, the DNA damage marker. Ponceau staining of the immunoblots is provided to illustrate equal loading of protein.

Cdt1 phosphomutant proteins Induce Lower Levels of Re-replication in Comparison to WT Cdt1

While we observed less activation of the DNA damage marker phospho-Chk1 in cells with overexpressed Cdt1 phosphomutant proteins (S93A, T152A, and 2A), these results only indicate lower levels of DNA damage pathway activation. These results do not necessarily directly show lower levels of re-replication. Therefore, in order to examine re-replication we performed flow cytometry. After growing all five cell lines (GFP, WT, S93A, T152A, and 2A) for three days with Dox, we labeled cells with EdU to measure DNA synthesis and DAPI to measure the DNA content in the cells. As seen in Figure 5A, cells which underwent re-replication were gated in the box marked "R", since these cells have DNA content (DAPI) greater than 4C (i.e. more than twice the DNA content after DNA replication). The percentage of cells in each phase of the cell cycle (G1, S, G2) and those which underwent re-replication (R) were quantified (Figure 5B). Additionally, the percentage of cells which underwent re-replication was graphed for each cell line (Figure 5C). Currently, an immunoblot analysis on these samples is being performed. However, a representative immunoblot of cells which gave similar results in flow cytometry is provided in Figure 5D. This immunoblot indicates that ectopic expression of Cdt1 WT and 2A was similar with S93A ~2 times less ectopic expression than WT. Ectopic expression of Cdt1 T152A was under endogenous levels.

GFP cells treated with MLN4924 served as a positive control because it acts as an ubiquitin ligase inhibitor, inhibiting Cdt1 from being ubiquitinated and degraded. Stabilization of endogenous Cdt1 was observed in the immunoblot analysis in lane 2 (Figure 5D). The positive control, GFP cells with MLN4924, had the highest percentage of cells which re-replicated (14.57%), with cells with overexpressed ectopic WT Cdt1 following behind with 8.52% of the cell population which re-replicated (Figure 5C). Interestingly, cells with overexpressed Cdt1

phosphomutant proteins exhibited markedly reduced levels of re-replication. The percentage of cells which re-replicated was 1.61% for the overexpression of Cdt1 S93A, when it had ~2 fold lower ectopic expression in comparison to WT ectopic expression. Conclusions cannot be made about T152A due to low ectopic expression of the mutationally-altered protein. The percentage of cells which re-replicated was 0.47% for the overexpression of Cdt1 2A, when it had similar levels of ectopic expression as WT. The percentage of cells which re-replicated in the phosphomutant cells, specifically S93A and 2A, is near the percentage of cells which re-replicated for the negative control GFP (0.65%) indicating that these phosphomutant proteins induced less re-replication than WT Cdt1 (8.52%). In accordance with our previous results, Cdt1 S93A allele caused more re-replication than the other two phosphomutant proteins. It is important to note that Cdt1 2A had higher percentages of cells in G1 phase in comparison to Cdt1 WT or GFP. It is possible that cells with this mutationally-altered Cdt1 protein are stuck in G1 phase and unable to go through S phase or re-replicate. Overall, while 2A was ectopically expressed at the same level as WT Cdt1, it induced much lower levels of rereplication. It is likely that S93A is a least a major contributor to this effect. To confirm these results, we will be setting up two more replicate experiments and will quantify the standard error in the percentage of cells re-replicating for each Cdt1 allele.

A)

GFP | WT | GFP +MLN4924

S93A | T152A | 2A

DNA Synthesis (y-axis)

DNA Content (x-axis)
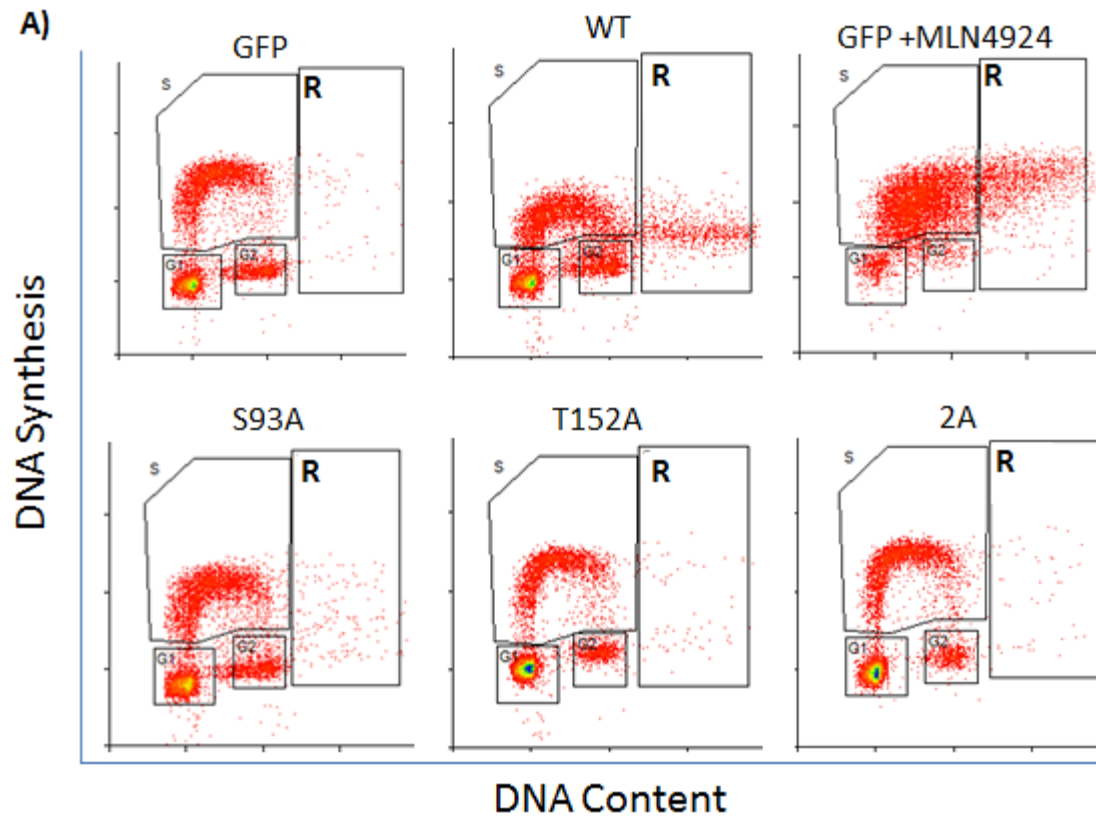
B)

	Count	G1	S	G2	R
GFP	9762	39.11%	45%	13.02%	0.65%
WT	10644	35.79%	37.06%	14.51%	8.52%
S93A	9837	39.22%	43.11%	12.97%	1.61%
T152A	9840	56.9%	32.37%	9.55%	0.6%
2A	9860	52.17%	38.01%	8.67%	0.47%
GFP +MLN4924	10395	6.29%	73.63%	2.21%	14.57%
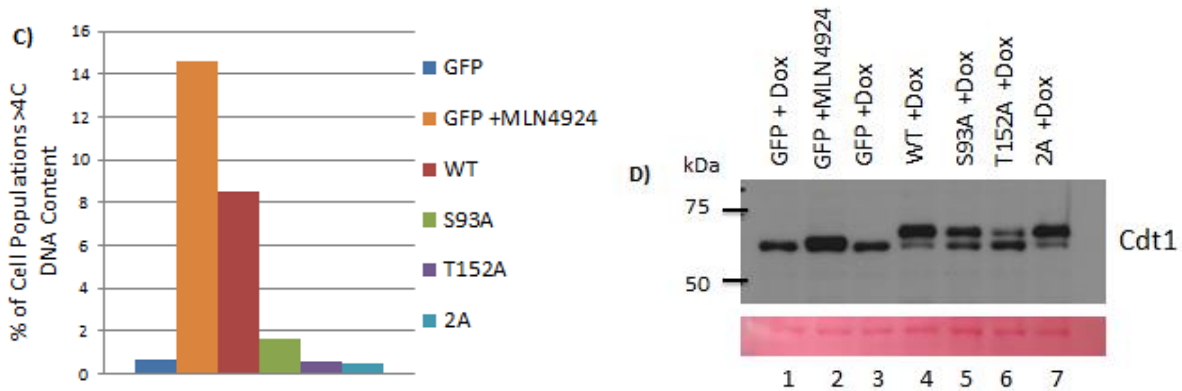
Figure 5. Re-replication Induction by Cdt1 Overexpression. A) U2OSa cells were treated with doxycycline for 72 hour to overexpress either ectopic Cdt1 or GFP (control). MLN4924 serves as a positive control for re-replication. Cells were labeled for 30-45 minutes with EdU to detect DNA synthesis and analyzed by flow cytometry with DAPI for DNA content. Flow cytometry gating for re-replication is indicated in the schematic with an "R". **B)** Percentage of the total population in each cell cycle phase is quantified and in **C)** re-replication percentages are graphically represented. **D)** Representative immunoblot illustrating ectopic overexpression and endogenous expression of Cdt1 (WT and phosphomutants). Ponceau staining of the immunoblot is provided to illustrate equal loading of protein.

Discussion

In this study we generated human U2OS cell lines expressing ectopic Cdt1 WT, S93A, T152A, or 2A to study the cellular phenotypes of two phosphorylation sites of Cdt1. Previous studies have shown that multiple N-terminal phosphorylation sites on Cdt1 are crucial for its regulation and activity (Chandrasekaran et al., 2011). However, phosphorylation regulation on other regions of Cdt1 is not fully understood. Thus, we chose to study these recently discovered phosphorylation sites at S93 and T152 (Hornbeck et al., 2012; Mertins et al., 2013; Sharma et al., 2014). Studying the phenotypes of these phosphorylation mutants will enable us to determine whether these two phosphorylation sites play a role in Cdt1 function. Since Cdt1 is involved in preparing the genome for replication during the cell cycle, it follows that Cdt1 has also been implicated in many cancers. Studies have found that Cdt1 is highly expressed in many cancers including non-small-cell lung carcinoma and colon cancer (Bravou, Nishitani, Song, Taraviras,

& Varakis, 2005; Kouloukoussa & Nishitani, 2004). Additionally, MAPKs and Cdks, which are involved in regulating Cdt1 function, are deregulated in many cancers (Dhillon, Hagan, Rath, & Kolch, 2007; Tsihlias, Kapusta, & Slingerland, 1999). Thus, it is likely that phosphorylation regulation of Cdt1 is likely to be deregulated in many cancers as well. Furthermore, one model of cancer development proposes that activated oncogenes disrupt cellular proliferation by causing replication stress during the replication process (Halazonetis, Gorgoulis, & Bartek, 2008). Due to Cdt1's involvement in the pre-replication complex, it may play a role in carcinogenesis. Thus, exploring how Cdt1 is regulated through phosphorylation will also contribute to our understanding of the biological underpinnings of cancer and other Cdt1-related diseases.

We first created human U2OS cells lines harboring ectopic Cdt1 (WT, S93A, T152A, and 2A) under a doxycycline inducible expression system. The immunoblot demonstrated all three phosphomutant cell lines are under the control of doxycycline and stably express ectopic Cdt1 in the presence of Dox. One caveat of using doxycycline inducible cells is the inability to remove endogenous WT Cdt1. Thus, we used a high doxycycline concentration to induce overexpression of ectopic Cdt1. However, using the same doxycycline concentration causes variable levels of ectopic Cdt1 overexpression across phosphomutant cell lines, especially for the T152A mutant cell line. A possible reason behind differing levels of ectopic expression is that the vector may express better in some of the cell lines than others. Additionally, the proteins may have different stabilities or kinetics. Currently, tests are being performed on the T152A mutant cell line to determine whether the Cdt1 protein is unstable or if the cell line needs to be reestablished. To account for differences in ectopic expression, we normalized to tubulin levels in subsequent experiments.

To study whether the phosphorylation sites at S93 and T152 affect Cdt1 function, we examined the cellular phenotype of WT Cdt1 and the three phosphomutants. Colony forming assays illustrated variable levels of colony formation in cells with overexpressed ectopic Cdt1 WT and phosphomutant protein. When ectopic WT Cdt1 was overexpressed at higher levels than endogenous, colony formation was reduced to significantly lower levels. While WT Cdt1 was the most toxic to cells, Cdt1 S93A was slightly less toxic than WT Cdt1, followed by a lower toxicity caused by Cdt1 2A. One issue we encountered was the ectopic expression of Cdt1 T152A. While the immunoblot was equally loaded with protein, the ectopic expression of Cdt1 T152A was near or below endogenous levels of Cdt1 during replicate experiments. As mentioned previously, this could be due to low expression by the vector. Future studies will utilize a replicate cell line with ectopic Cdt1 T152A or we will employ an alternative expression approach. Thus, conclusions cannot be made about T152A mutationally-altered protein. However, our results indicate that S93A and 2A mutational alterations of Cdt1 are less functional than WT Cdt1. Thus, phosphorylation at this site or the proper amino acid (either serine or threonine) is required for full Cdt1 activity and function.

Since it is known that Cdt1 can cause relicensing and re-replication, we were interested in whether the cell death we observed in the colony formation assays was due to DNA damage (Vaziri et al., 2003). We examined the expression of phospho-Chk1, a marker for ATR DNA damage pathway activation. Phosphorylation of Chk1 occurs in response to genotoxic stress and blocked DNA replication (Piwnica-Worms, 2001). While the immunoblot demonstrated equal levels of Cdt1 expression at 24 and 42 hours, phospho-Chk1 expression was more discernible at 24 hours. Immunoblot analysis at 24 hours indicated cells with ectopic phosphomutant proteins activated phospho-Chk1 in lower quantities than in the WT Cdt1 cells. When ectopic Cdt1 S93A

expression was slightly less than ectopic WT Cdt1, cells with ectopic Cdt1 S93A activated phospho-Chk1 at a lower level than cells with ectopic WT Cdt1. Additionally, when ectopic Cdt1 S93A was expressed slightly higher than Cdt1 2A, phospho-Chk1 was activated at a higher level in cells with Cdt1 S93A. While the ectopic expression of Cdt1 varies between WT and mutant cell lines, the ectopic expression of Cdt1 phosphomutant proteins likely fail to activate DNA damage response pathways to the same extent as ectopic expression of WT Cdt1. Furthermore, the reduction in phospho-Chk1 activation is seen to a large extent with the ectopic expression of Cdt1 S93A, in comparison to Cdt1 2A, indicating that the phosphorylation site at S93 is required for full Cdt1-induced DNA damage. Further studies are needed to examine phospho-Chk1 activation while accounting for differences in ectopic expression levels. This experiment will be replicated in the future because positive control samples (UV-treated control GFP cells) activated phospho-Chk1 at a lower level than expected and negative control samples (GFP cells + and - Dox) activated phospho-Chk1 at higher levels than expected. These results may have been observed due to suboptimal culture conditions and technical difficulties in UV treatment.

While expression of phospho-Chk1 demonstrates the activation of the ATR DNA damage pathway, it does not provide information on replication stress specifically. To determine whether the phosphomutant cells cause differential levels of re-replication, we performed flow cytometry on Cdt1 WT and phosphomutant cell lines. Flow cytometry analysis demonstrated that a lower percentage of cells underwent re-replication with the overexpression of Cdt1 S93A and 2A phosphomutant proteins than with overexpressed WT Cdt1. (WT Cdt1 was ectopically overexpressed at similar levels to ectopic Cdt1 2A and was slightly higher expressed than Cdt1

S93A.) These results indicate that the phosphomutant protein 2A and most likely S93A are less functional in terms of re-replication than WT Cdt1.

One interesting result in flow cytometry analysis also provides insight to how these two phosphorylation sites may affect Cdt1 function. Cells with overexpressed Cdt1 2A had higher percentages of cells in G1 phase during flow cytometry, compared to cells with overexpressed Cdt1 WT and Cdt1 S93A. It is possible that the phosphorylation mutation at S93A and T152A or the absence of the original amino acids (serine or threonine) prohibit cells from transitioning into S phase.

There could be a number of reasons for this: First, mutating these phosphorylation sites may alter Cdt1 degradation or detachment from the pre-replication complex or may inhibit pre-replication complex formation. Phosphorylation at T29 of Cdt1 is controlled by cyclin-dependent kinases (Cdks), which enables binding of SCF-Skp2 E3 ubiquitin ligase and subsequent ubiquitination and degradation of Cdt1 (Nishitani et al., 2006; Sugimoto et al., 2004). S93A and T152A phosphorylation may also be regulated by Cdks or MAPKs since S93 and T152 are followed by prolines and this site may be involved in SCF^{Skp}-mediated degradation (Chandrasekaran et al., 2011; Nishitani et al., 2006). Second, phosphorylation of S93A may affect MCM helicase loading. Interactions between Cdt1 and Cdc6 or with ORC subunits, necessary for the loading of MCM helicase, have not been identified. Thus, inhibiting phosphorylation on T152 or S93 may hinder Cdt1 to bind with other key proteins, preventing re-replication. Furthermore, these two phosphorylation sites may be involved in Cdt1 function in mitosis. Future studies will explore if these mutationally-altered Cdt1 proteins affect binding to Hec1 component of Ndc80 complex. We currently do not know when in the cell cycle these sites become phosphorylated. It is possible that phosphorylation occurs before mitosis or halfway

through mitosis, when Cdt1's inhibitor, Geminin, becomes degraded, and Cdt1 levels start to increase (McGarry & Kirschner, 1998). It has been found that Cdt1 starts to license origins during telophase (Dimitrova, Prokhorova, Blow, Todorov, & Gilbert, 2002).

The aforementioned results indicate that S93 and T152 sites play an important role in the function of Cdt1. Mutating both S93 and T152 such that these sites are unable to undergo phosphorylation impairs the activity of Cdt1. Thus, phosphorylation at these sites is required for full Cdt1 function. Additionally, S93 is likely to have a major contribution to Cdt1's function. Future studies will focus on the molecular underpinnings of these phosphorylation sites, such as the ability of Cdt1 to bind to other proteins. Additionally, we will examine which kinases phosphorylate these sites and at what point in the cell cycle these sites become phosphorylated.

Acknowledgements

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