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The p150N domain of chromatin assembly factor-1 regulates Ki-67 accumulation on the mitotic perichromosomal layer

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ABSTRACT Chromatin assembly factor 1 (CAF-1) deposits histones during DNA synthesis. The p150 subunit of human CAF-1 contains an N-terminal domain (p150N) that is dispensable for histone deposition but promotes the localization of specific loci (nucleolar-associated domains [NADs]) and proteins to the nucleolus during interphase. One of the p150N-regulated proteins is proliferation antigen Ki-67, whose depletion also decreases the nucleolar association of NADs. Ki-67 is also a fundamental component of the perichromosomal layer (PCL), a sheath of proteins surrounding condensed chromosomes during mitosis. We show here that a subset of p150 localizes to the PCL during mitosis and that p150N is required for normal levels of Ki-67 accumulation on the PCL. This activity requires the sumoylation-interacting motif within p150N, which is also required for the nucleolar localization of NADs and Ki-67 during interphase. In this manner, p150N coordinates both interphase and mitotic nuclear structures via Ki67.

INTRODUCTION

Eukaryotic chromosomes must condense for segregation during mitosis and then decondense and readopt interphase configurations in the next cell cycle. However, mechanisms that link higher-order mitotic and interphase genome organization remain poorly understood. Of note, cellular heterochromatin is highly enriched at specific sites in interphase nuclei, at the nuclear lamina, in pericentric foci, and in perinucleolar regions (reviewed in Politz et al., 2016). Therefore major questions relate to how these loci are relocalized after each mitosis and what mitotic molecules might aid in this.

Nucleolar-associated domains (NADs) are genomic loci that interact frequently with the nucleolar periphery (Németh et al., 2010; van Koningsbruggen et al., 2010; Padeken and Heun, 2014; Matheson and Kaufman, 2015; Pontvianne et al., 2016). NADs are highly enriched for repetitive DNA satellites and histone modifications associated with heterochromatic silencing, such as H3K27me3, H3K9me3, and H4K20me3 (Németh et al. 2010; Politz et al. 2016). One protein shown to regulate NAD localization is the p150 subunit of chromatin assembly factor 1 (CAF-1; Smith et al., 2014). CAF-1 is a highly conserved three-subunit protein complex that deposits histone $(H3/H4)_2$ tetramers onto replicating DNA during S phase of the cell cycle (Smith and Stillman, 1989; Kaufman et al., 1995; Krude, 1995) and is particularly important for DNA replication and maintenance of histone marks at heterochromatic loci (Reese et al., 2003; Quivy et al., 2004, 2008; Sarraf and Stancheva, 2004; Houlard et al., 2006; Dohke et al., 2008; Huang et al., 2010; Baldeyron et al., 2011). In addition to these functions, the N-terminal domain of p150 (p150N) regulates the association of NADs to the perinucleolar region and also regulates the nucleolar localization of several proteins, including the proliferation antigen Ki-67 (Smith et al., 2014).

Ki-67 also controls heterochromatin organization (Sobecki et al., 2016) and affects chromosome condensation (Takagi et al., 1999; Kametaka et al., 2002). Ki-67 is highly expressed in proliferating cells and minimally expressed in quiescent cells (Gerdes et al., 1983, 1984), and meta-analyses of numerous clinical studies have validated
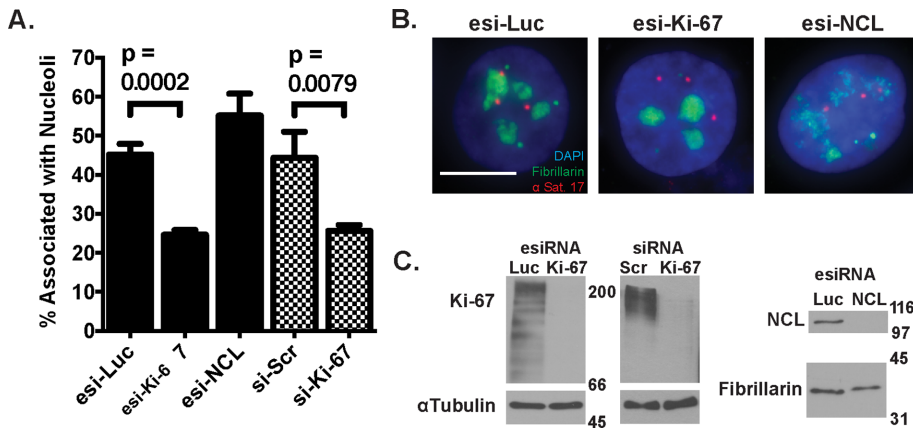
FIGURE 1: α-Satellite associations with nucleoli are reduced upon depletion of Ki-67 but not nucleolin. (A) Immuno-FISH analyses of the association between α-satellite DNA from chromosome 17 (αSat 17, red) and fibrillarin (green) was performed in HeLa cells transfected for 72 h with in vitro–diced esiRNAs targeting luciferase, Ki-67, or NCL (black bars) or with synthetic duplex siRNAs (checked bars) targeting Ki-67 or a scrambled sequence control (si-Scr). The percentage of αSat 17 alleles colocalized with fibrillarin is indicated, with means and SD error bars from three replicate experiments. *p* values comparing association frequencies in cells treated with esi-Luc (669 alleles) vs. esi-Ki-67 (573 alleles) and si-SCR (540 alleles) with si-Ki-67 (567 alleles) are indicated. (B) Representative FISH images of cells analyzed in A. Scale bar, 10 μm. (C) Immunoblot analyses of cells from A. Blots were probed with antibodies recognizing Ki-67, α-tubulin (loading control), NCL, or fibrillarin (loading control), as indicated.

Ki-67 as a prognostic marker in grading tumors (Luo *et al.*, 2015; Pezzilli *et al.*, 2016; Pyo *et al.*, 2016; Richards-Taylor *et al.*, 2016). Similar to Ki-67, p150 is also highly expressed in proliferating cells and minimally expressed in quiescent cells and has been proposed as an alternative cellular proliferation marker in clinical studies (Polo *et al.*, 2004).

Recent studies demonstrate that Ki-67 is required for formation of the perichromosomal layer (PCL; Booth *et al.*, 2014; Sobecki *et al.*, 2016), a layer of proteins that surrounds all condensed chromosomes during mitosis (reviewed in Van Hooser *et al.*, 2005). At the PCL, Ki-67's status as a large, charged protein that possesses "surfactant"-like properties keeps individual mitotic chromosomes dispersed rather than aggregated upon nuclear envelope disassembly, thereby ensuring normal kinetics of anaphase progression (Cuylen *et al.*, 2016). Similarly, acute depletion experiments show that Ki-67 is important for maintaining mitotic chromosome structure and function (Takagi *et al.*, 2016). Because p150N regulates interphase Ki-67 localization, we investigated whether p150N also affects Ki-67 localization during mitosis.

RESULTS AND DISCUSSION

Ki-67 regulates α-satellite localization to the perinucleolar region

Recent studies suggest that Ki-67 regulates repetitive DNA localization to the nucleolus. Ki-67 depletion decreased the nucleolar association of a LacO array proximal to the rDNA repeats on chromosome 13 (Booth *et al.*, 2014) and the centromeric histone variant CENP-A (Sobecki *et al.*, 2016). To determine whether Ki-67 also regulates satellite repeat association with the nucleolus, we transfected small interfering RNAs (siRNAs) into HeLa cells and performed immuno–fluorescent in situ hybridization (FISH) to visualize the position of α-satellite DNA from chromosome 17 (αSat 17) relative to the nucleolar protein fibrillarin. HeLa cells transfected with siRNAs directed against luciferase or a scramble control displayed ~45% of αSat 17 alleles associated with nucleoli, which represents a high-frequency interaction characteristic of a NAD locus (Németh *et al.*,

2010; van Koningsbruggen *et al.*, 2010; Smith *et al.*, 2014). In contrast, cells transfected with two different siRNA reagents directed against distinct regions of the Ki-67 mRNA displayed αSat 17 association frequencies to ~25% (Figure 1, A and B).

We also depleted HeLa cells of nucleolin (NCL), a nucleolar protein that primarily localizes to the perinucleolar region (Bugler *et al.*, 1982) and regulates transcription, folding, and assembly of rRNA (Ginisty *et al.*, 1998, 1999; Roger *et al.*, 2002; Rickards *et al.*, 2007). Depletion of NCL altered nucleolar morphology (Figure 1B), as previously reported (Ma *et al.*, 2007; Ugrinova *et al.*, 2007), but did not affect the association of αSat 17 with fibrillarin (Figure 1A). We conclude that in human cells, Ki-67 is required for efficient localization of NADs to nucleoli and that NCL is not required, at least for α-satellite–nucleolar interactions.

Our results with human nucleolin depletion are somewhat different from those obtained in a recent study of nucleolin's contribution to NAD localization in the plant *Arabidopsis thaliana* (Pontvianne *et al.*, 2016). As in our experiments (Figure 1B), cells from nucleolin mutant (*nuc1*) plants also showed altered nucleolar morphology. However, deep sequencing of purified nucleoli revealed altered NAD profiles in *nuc1* cells, including decreased nucleolar association of telomeric heterochromatin, coinciding with a 30% loss of telomere length on all chromosomes. It is possible that there are altered NAD associations in nucleolin-depleted human cells that we could not have detected using an α-satellite probe specific for chromosome 17. Alternatively, there is precedence for species-specific interactions mediated by nucleolin. For example, depletion of nucleolin increases centromere–nucleolar interactions in *Arabidopsis* (Pontvianne *et al.*, 2016) but decreases such interactions in *Drosophila* (Padeken *et al.*, 2013).

Of interest, decreased telomere lengths were also observed in *Arabidopsis fas1* and *fas2* mutants (Pontvianne *et al.*, 2016), which lack the homologues of the human p150 and p60 subunits of CAF-1. Another study found that *fas1* and *fas2* mutants also display decreased copy number of the 45S rRNA genes, along with increased nucleolar association of the remaining 45S rRNA genes (Pontvianne *et al.*, 2013). Note that human p150 and *Arabidopsis* Fas1 share 29.8% amino acid identity, but Fas1 completely lacks the p150N domain, the N-terminal 310 amino acids of p150 previously shown to regulate human nucleolar structure (Smith *et al.*, 2014). Together these data suggest that p150/Fas1 regulates nucleolar structure in both plants and mammals but also that there are marked mechanistic differences in how this regulation occurs in different organisms. We also note that a higher percentage of the genome is nucleolar associated in human cells (Dillinger *et al.*, 2016) than in plant cells (Pontvianne *et al.*, 2016).

A subset of p150 colocalizes with Ki-67 foci during mitosis and early G1 phases

Ki-67 has three distinct nuclear localization patterns, which appear at distinct cell-cycle periods (see later discussion of Figure 5A). First, Ki-67 localizes to the nucleolus during interphase (Verheijen *et al.*, 1989a; Traut *et al.*, 2002; Cheutin *et al.*, 2003), and a subset colocalizes with p150 at the perinucleolar region (Smith *et al.*, 2014).
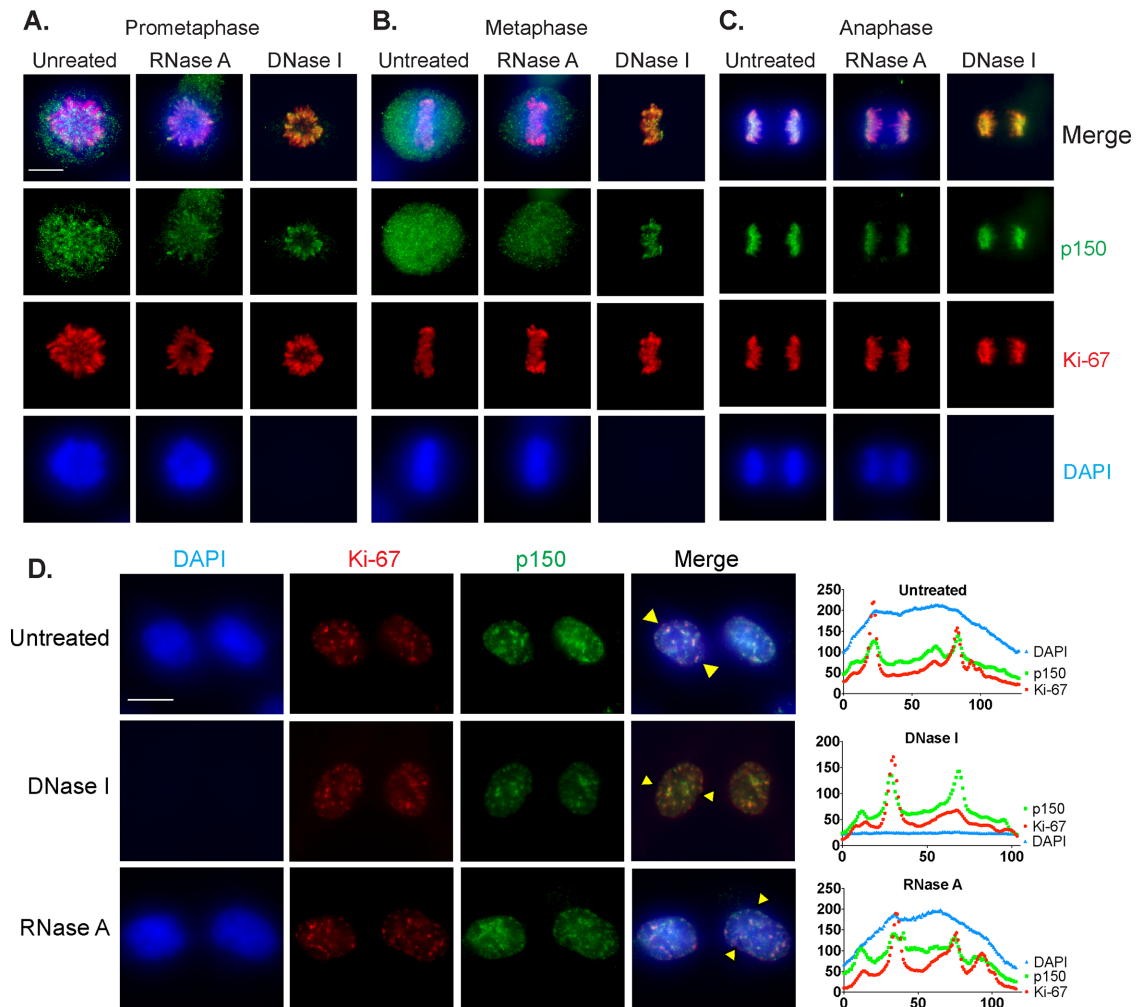
FIGURE 2: A subset of p150 localizes to the PCL during mitosis and to Ki-67 foci during early G1 of interphase. RPE1-hTERT cells were either untreated or digested with RNase A or DNase I as indicated and then high-salt extracted. Cells were stained with DAPI to detect DNA (blue) and with antibodies recognizing p150 (green) and Ki-67 (red). Cells from prophase (A), metaphase (B), and anaphase (C). Note that DNase I–treated cells lack DAPI staining. Scale bar, 10 μm. (D) p150 colocalizes with Ki-67 foci during early G1. RPE1-hTERT cells were treated and stained as indicated. Pairs of recently divided cells featuring hundreds of Ki-67 foci characteristic of early G1 are shown. Line scans (right) of individual cells (yellow triangles in merged images) were used to assess colocalization of p150 with Ki-67 signal. Scale bar, 10 μm.

Second, Ki-67 localizes to the PCL during mitosis (Gerdes *et al.*, 1983; Gerdes *et al.*, 1984; Verheijen *et al.*, 1989b). In contrast to the chromosome-associated state of mitotic Ki-67, mitotic phosphorylation evicts the majority of CAF-1 from mitotic chromosomes and inhibits its nucleosome assembly activity (Matsumoto-Taniura *et al.*, 1996; Marheineke and Krude, 1998; Murzina *et al.*, 1999; Keller and Krude, 2000). However, mass spectrometric analyses suggested that a mitotic chromosome–associated subset of p150 existed (Ohta *et al.* 2010), but its relationship to the PCL was not directly tested. To analyze this, we took advantage of the fact that Ki-67 localization to the PCL remains visibly unchanged even after extraction with 2 M NaCl and treatment with either RNase A or DNase I (Sheval and Polyakov, 2008), indicating that the structural stability of the PCL is independent of the chromatin it surrounds. We performed immunofluorescence on RPE1-hTERT cells that were digested with either RNase A or DNase I or mock digested and then high-salt extracted (Supplemental Figure S1). As previously reported, Ki-67 remained on the PCL throughout mitosis even after DNA or RNA digestion, as shown for prophase (Figure 2A), metaphase (Figure 2B), and anaphase (Figure 2C). As expected, p150, as well as Ki67, could be easily detected on anaphase chromosomes presumably because at that stage, the mitotic phosphorylations of CAF-1 are removed, triggering bulk reassociation with chromatin and reactivation of nucleosome assembly activity (Keller and Krude, 2000). In contrast, during prophase or metaphase, a large amount of p150 evicted into the nucleoplasm made it difficult to determine whether p150 localized to the PCL. The combination of high-salt extraction and DNase I digestion, however, revealed that a subset of p150 localized to the PCL during all phases of mitosis.

The third Ki-67 localization pattern occurs after cytokinesis and early in G1 phase. At this stage, Ki-67 localizes to hundreds of distinct foci (Isola *et al.*, 1990; du Manoir *et al.*, 1991), which colocalize with heterochromatic satellite repeats (Bridger *et al.*, 1998). These foci are transient and appear to be intermediates in the process of
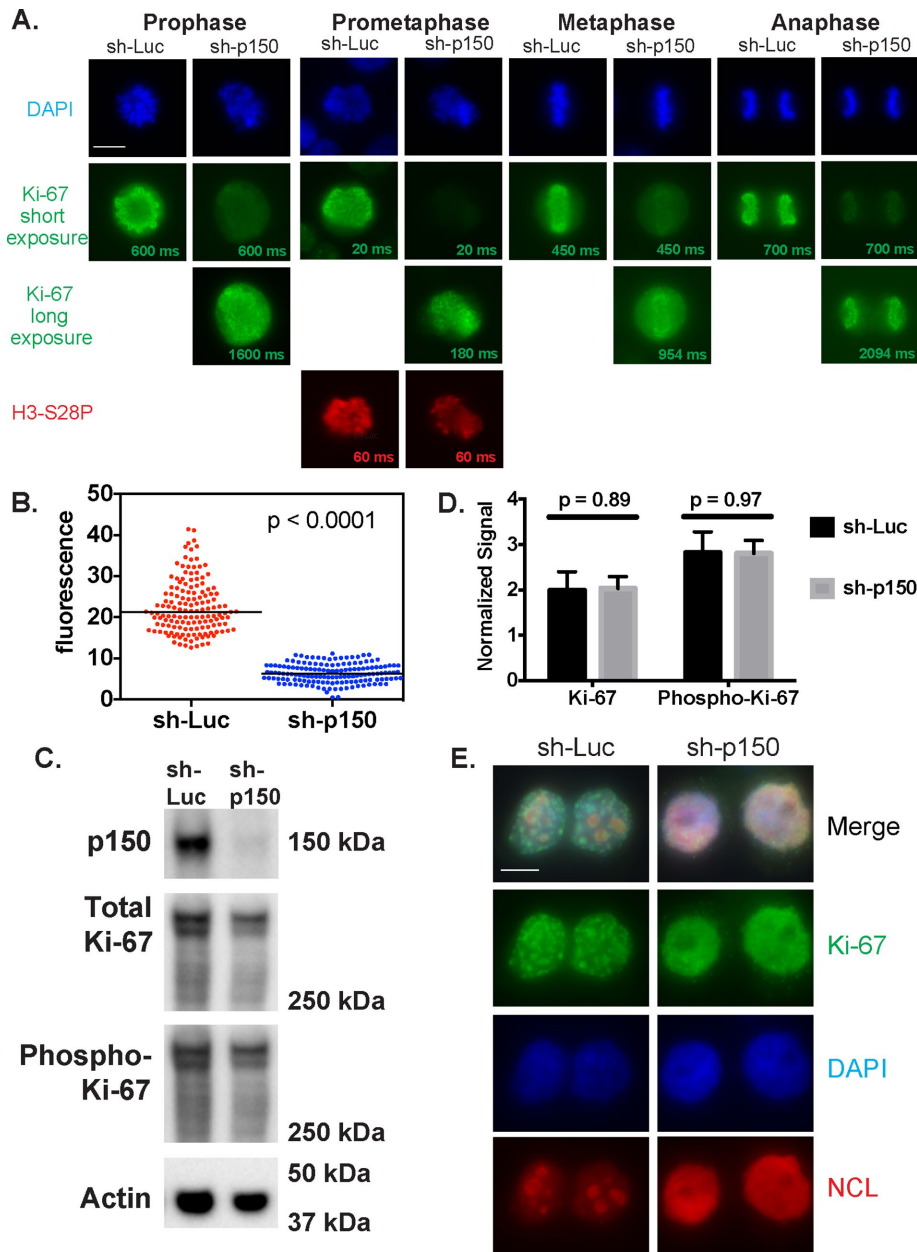
FIGURE 3: p150 regulates Ki-67 localization during mitosis and early G1 phase. (A) HeLa S3 cells from the indicated cell cycle stages expressed an shRNA directed against luciferase (sh-Luc) or p150 (sh-p150) for 72 h and were stained with DAPI to visualize DNA (blue) and antibodies against Ki-67 (green). Exposure times for Ki67 are indicated on each image; in the sh-p150–expressing cells, different exposure times are shown to illustrate reduced Ki-67 accumulation on the PCL. As a positive control for antibody accessibility, cells in prometaphase were also stained with antibodies recognizing the mitotic marker histone H3-S28-phosphate (red). Scale bar, 10 μm. (B) Quantified corrected total cellular fluorescence of cells from three biological replicate experiments of cells expressing sh-luc (red, $N = 150$) or sh-p150 (blue, $N = 147$). (C) Immunoblot analysis of extracts from shRNA-expressing cells described in A arrested in mitosis (12 h in 100 ng/ml nocodazole, followed by shaking off mitotic cells). Blots were probed with antibodies recognizing p150 (top), Ki-67 (upper middle), phospho-Ki-67 (lower middle), and actin (loading control; bottom). Numbers on the right indicate migration of marker proteins in kilodaltons. (D) Quantification of the Ki-67 and phospho-Ki-67 blots from C normalized to actin signal ($N = 3$). Quantification was performed using the Bio-Rad ChemiDoc system. (E) HeLa S3 cells that expressed an shRNA directed against luciferase (sh-Luc) or p150 (sh-p150) for 72 h were synchronized in mitosis (12 h in 100 ng/ml, nocodazole followed by shaking off), and released into drug-free medium for 2 h to enrich for early G1 cells. Cells were stained with DAPI to visualize DNA (blue) and antibodies against Ki-67 (green) and NCL (red). Scale bar, 10 μm.

reformation of nucleoli as cells transition from mitosis to interphase (Saiwaki *et al.*, 2005). Because a subset of p150 is in the mitotic PCL (Figure 2, A–C), we sought to determine whether p150 also colocalized with early G1 Ki-67 foci. To do this, we performed immunofluorescence on early G1 RPE1-hTERT cells digested with nucleases or mock digested as previously described (Figure 2D). In all three conditions, a subset of p150 foci in early G1 cells colocalized with Ki-67 foci, as indicated by line scan analyses. These data suggest that a subset of p150 travels together with Ki67 during exit from mitosis, as PCL components transit toward reforming nucleoli.

p150 regulates Ki-67 accumulation on the perichromosomal layer

Our previous work demonstrated that p150 is required for normal steady-state accumulation of Ki-67 in the nucleolus during interphase (Smith *et al.*, 2014). Because Ki-67 is essential for the formation of the PCL in mitotic cells (Booth *et al.*, 2014; Sobecki *et al.*, 2016), we tested whether p150 also regulated Ki-67 localization during mitosis. Via immunofluorescence, we examined Ki-67 distribution in HeLa S3 cells expressing an inducible short hairpin RNA (shRNA) directed against either luciferase (Luc) or p150 (Figure 3A). As expected, we found that Ki-67 robustly associated with the PCL during all phases of mitosis in the negative control cells expressing sh-Luc. In contrast, cells expressing sh-p150 displayed less Ki-67 staining on the PCL, as demonstrated in the exposure times matched with the sh-Luc samples. When exposure times were increased, Ki-67 was detected on the PCL, indicating that the PCL was not entirely disassembled upon p150 depletion. When the Ki-67 fluorescence was quantified in prometaphase cells from three biological replicates, cells expressing sh-p150 displayed, on average, a 3.5-fold decrease in fluorescence intensity compared with the control cells expressing sh-Luc (Figure 3B). To test whether this effect of p150 depletion could result from global masking of epitopes on the PCL, we costained some samples with an antibody recognizing the mitotic histone modification H3-S28ph (Goto *et al.*, 1999, 2002). As shown in the prometaphase cell in Figure 3A, this antibody stained cells expressing sh-Luc or sh-p150 equally well, suggesting that p150 regulates Ki-67 accumulation on the PCL rather than affecting overall epitope accessibility on mitotic chromosomes.

To explore how p150 may be regulating Ki-67 localization, we tested whether p150 is required for maintaining steady-state levels of Ki-67. Our previous work demonstrated that Ki-67 steady-state levels were not affected upon p150 depletion in asynchronous cells (Smith *et al.*, 2014). To determine whether p150 regulates the steady-state protein levels of Ki-67 during mitosis, we collected extracts from mitotic-arrested cells expressing sh-Luc or sh-p150 (Supplemental Figure S2) and analyzed them by immunoblotting (Smith *et al.*, 2014; Figure 3, C and D). Total levels of Ki-67 in mitotic samples normalized to actin signals were not significantly changed upon p150 depletion (Figure 3, C and D), indicating that p150 is not required for maintaining steady-state levels of Ki-67 during mitosis. At the beginning of mitosis, Ki-67 localization to the PCL occurs in conjunction with hyperphosphorylation of the Ki-67 protein (MacCallum and Hall, 1999; Endl and Gerdes, 2000; Takagi *et al.*, 2014). These phosphorylations are important for PCL localization, as treatment of mitotic cells with protein kinase inhibitors results in dephosphorylation of Ki-67 and relocalization of Ki-67 away from the PCL to distinct nuclear foci (MacCallum and Hall, 1999). Therefore we tested whether p150 regulates Ki-67's mitotic phosphorylation status by reprobing the immunoblots of mitotically arrested cell extracts with antibodies that specifically recognize Ki-67 phosphorylated on Cdk consensus sites within the Ki-67 internal repeat structure (Takagi *et al.*, 2014). However, we detected no statistically significant changes upon p150 depletion (Figure 3, C and D). Therefore p150 does not appear to regulate the steady-state levels or phosphorylation of Ki-67 during mitosis.

p150 regulates the formation of Ki-67 foci in early G1 phase

Because p150 regulates the localization of Ki-67 to the nucleolus during interphase (Smith *et al.*, 2014) and to the PCL during mitosis (Figure 3A), we wanted to examine whether p150 is also required for the formation of the punctate Ki-67 foci seen at the beginning of G1 phase. To answer this question, we induced expression of sh-Luc or sh-p150 for 60 h and then synchronized cells by adding 100 ng/ml nocodazole for the final 12 h of shRNA expression. After vigorously shaking mitotic cells off the plate, we washed cells three times in phosphate-buffered saline (PBS) and released them into nocodazole-free medium. Based on fluorescence-activated cell sorting of a time course after release (Supplemental Figure S3), G1 cells were collected at 120 min postrelease and stained with antibodies directed against Ki-67 and NCL (Figure 3E). In the sh-Luc samples, hundreds of punctate Ki-67 foci were visible, and NCL was detected within the newly reformed nucleoli. In contrast, sh-p150 samples featured disrupted NCL localization and dispersal of Ki-67 foci. Together our data suggest that the contribution of p150N to interphase Ki-67 and NCL localization (Smith *et al.*, 2014) results from events that begin in M and/or G1 phase. In this manner, p150 regulates Ki-67 localization throughout the cell cycle (see later discussion of Figure 5).

The SIM within the N-terminus of p150 is required for Ki-67 localization to the PCL

To map p150 domains required for regulating Ki-67 accumulation on the PCL, we used previously published HeLa cell lines stably expressing V5-epitope–tagged, shRNA-resistant, p150-derived transgenes (Smith *et al.*, 2014). These V5-transgene cell lines were acutely depleted of endogenously encoded p150 via lentiviral expression of sh-p150 for 72 h before immunofluorescence analysis of Ki-67.
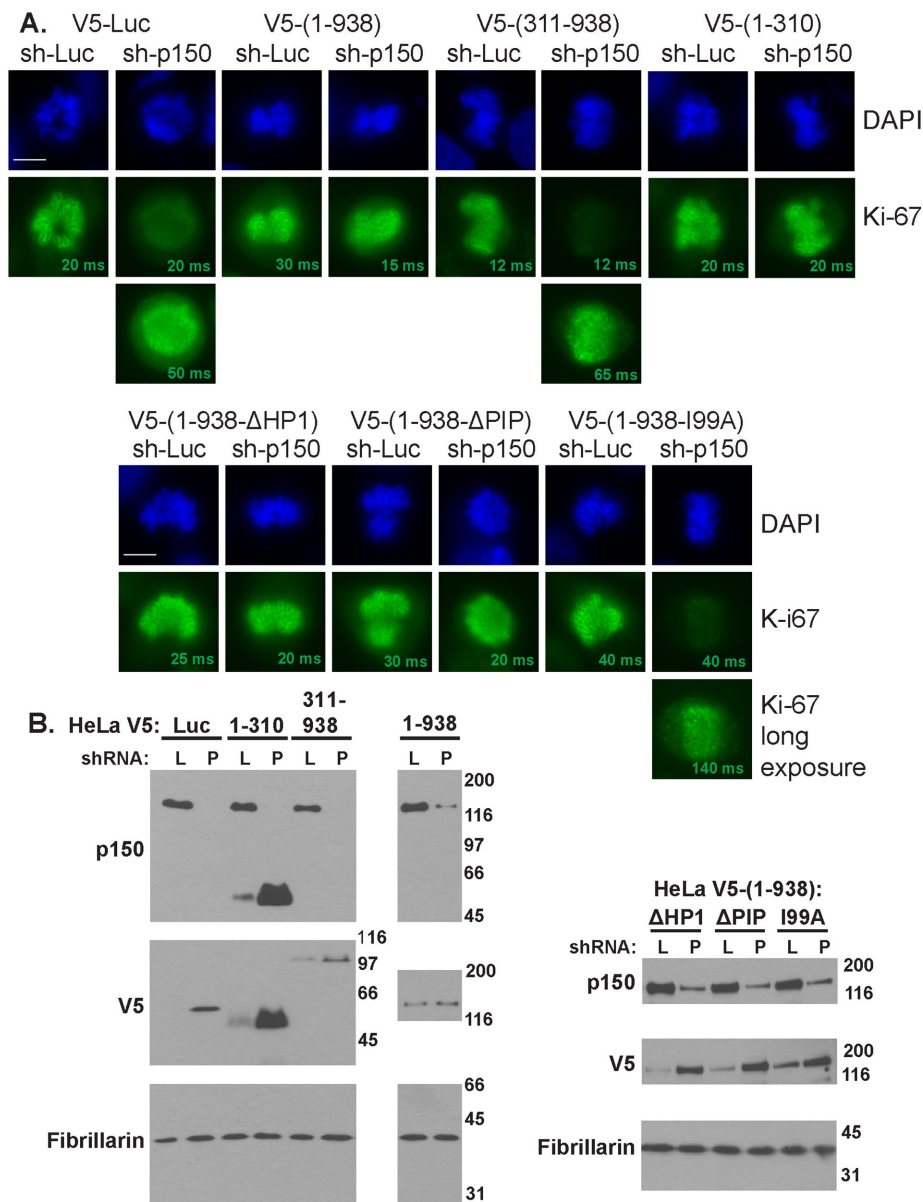
The C-terminal two-thirds of p150 serves as the scaffold for binding the other two subunits of the CAF-1 complex and thereby

is essential for CAF-1's nucleosome assembly activity (Kaufman *et al.*, 1995; Takami *et al.*, 2007). In contrast, p150's N-terminus is dispensable for nucleosome assembly (Kaufman *et al.*, 1995) but is necessary and sufficient to maintain Ki-67 localization to the nucleolus during interphase (Smith *et al.*, 2014). We found that depletion of p150 in HeLa cells expressing either luciferase or a p150 transgene encoding the C-terminus (amino acids 311–938) displayed reduced Ki-67 localization to the PCL (Figure 4A). In contrast, cells expressing either full-length p150 (amino acids 1–938) or only the N-terminus (p150N, amino acids 1–310) maintained normal amounts of Ki-67 on the PCL (Figure 4A). Therefore the p150 N-terminus regulates mitotic Ki-67 abundance in a manner independent of the chromatin assembly activity of the CAF-1 complex.

p150N contains several known interaction motifs, including a noncanonical PCNA-interacting peptide (PIP; Moggs *et al.*, 2000; Rolef Ben-Shahar *et al.*, 2009), a heterochromatin protein 1–binding domain (Murzina *et al.*, 1999), and a sumoylation-interacting motif (SIM; Uwada *et al.*, 2010; Sun and Hunter, 2012). Of note, the SIM in p150N is required for normal Ki-67 localization to the nucleolus during interphase (Smith *et al.*, 2014). We therefore determined whether transgenes encoding full-length p150 with mutations in foregoing three motifs described supported normal Ki-67 accumulation during mitosis. The cell lines expressing p150 transgenes with mutations in the PIP and HP1 domains maintained normal Ki-67 levels on the PCL. However, cells expressing a p150 transgene with a single amino acid mutation (I99A) that disrupts the SUMO-binding activity of the SIM (Uwada *et al.*, 2010) displayed reduced levels of Ki-67 on the PCL (Figure 4A). Together our data indicate that that the SIM motif within the N-terminus of p150 is required for the normal accumulation of Ki67 on the PCL during mitosis.

Together these data and our previous studies suggest a hierarchical relationship between p150N and Ki-67 in regulating nuclear architecture across the cell cycle (Figure 5, B–D). p150 and Ki-67 both colocalize during mitosis and interphase (Figure 2; Smith *et al.*, 2014), regulate NAD localization (Figure 1; Booth *et al.*, 2014; Smith *et al.*, 2014; Sobecki *et al.*, 2016), and have established roles in regulating heterochromatin (Quivy *et al.*, 2008; Sobecki *et al.*, 2016). It is noteworthy that p150 is required for the formation of punctate Ki-67 foci in early G1 of the cell cycle, as previous studies demonstrated that these foci colocalize with heterochromatic satellite repeats typically enriched within NADs (Bridger *et al.*, 1998). Because ablation of these foci results in mislocalization of NAD elements, we hypothesize that the function of these foci may be to guide heterochromatin-rich NADs back to the nucleolar periphery. However, additional evidence, such as the deep sequencing of the DNA elements contained within these foci, is required to further support this hypothesis.

p150N appears to function upstream of Ki-67 in this hierarchy, as p150N is required for efficient Ki-67 association with the PCL during mitosis (Figure 4) and with the nucleolus during interphase (Smith *et al.*, 2014). p150N is dispensable for nucleosome assembly by CAF-1, indicating that p150's role in regulating Ki-67 localization is independent of histone deposition. During both mitosis and interphase, the SIM within p150N is required for Ki-67 localization (Figure 4; Smith *et al.*, 2014), suggesting that this action involves an as-yet-unidentified sumoylated protein. These data suggest that future studies should explore the contributions of the p150N domain and its putative sumoylated binding partners to the relationship between Ki-67 and p150 in regulating mitotic and interphase nuclear structure.

A.

V5-Luc		V5-(1-938)		V5-(311-938)		V5-(1-310)	
sh-Luc	sh-p150	sh-Luc	sh-p150	sh-Luc	sh-p150	sh-Luc	sh-p150

DAPI

Ki-67

20 ms | 20 ms | 30 ms | 15 ms | 12 ms | 12 ms | 20 ms | 20 ms

50 ms — 65 ms

V5-(1-938-ΔHP1)		V5-(1-938-ΔPIP)		V5-(1-938-I99A)	
sh-Luc	sh-p150	sh-Luc	sh-p150	sh-Luc	sh-p150

DAPI

K-i67

25 ms | 20 ms | 30 ms | 20 ms | 40 ms | 40 ms

Ki-67 long exposure — 140 ms

B. HeLa V5: Luc | 1-310 | 311-938 | 1-938
shRNA: L P L P L P | L P

p150 — 200, 116, 97, 66, 45

V5 — 116, 97, 66, 45 ; 200, 116

Fibrillarin — 66, 45, 31

HeLa V5-(1-938): ΔHP1 ΔPIP I99A
shRNA: L P L P L P
p150 — 200, 116
V5 — 200, 116
Fibrillarin — 45, 31

FIGURE 4: The SIM within the N-terminal domain of p150 is required to maintain Ki-67 localization to the PCL. (A) HeLa cells in prometaphase expressing the indicated shRNA-resistant p150 transgene were infected with lentiviruses encoding the indicated shRNAs (sh-Luc or sh-p150) for 72 h and then stained with DAPI to visualize DNA (blue) and antibodies against Ki-67 (green). Note that upon expression of sh-p150, cells expressing luciferase (V5-Luc), the p150 C-terminus (V5-(p150-311-938)), or p150 with a I99A point mutation in the SIM motif required longer exposure times to detect Ki-67 on the PCL. Scale bar, 10 µm. (B) Immunoblots of cell extracts described in A expressing either sh-Luc (L) or ph-p150 (P). Blots were probed with antibodies recognizing p150 (top), V5 transgenes (middle), and fibrillarin (loading control; bottom). Note the depletion of endogenous p150 in sh-p150 lanes and that V5-tagged transgene expression often increased in these lanes, as we had previously (Smith et al., 2014).

MATERIALS AND METHODS
Cell culture
HeLa S3 cells containing the Trex CMV/TO promoters driving either sh-Luc or sh-p150 expression (Campeau et al., 2009) were maintained in RPMI medium with 5% tetracycline-free fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotic/antimycotic solution (Life Technologies). shRNA expression was induced by supplementing media with 2 µg/ml doxycycline for 72 h before fixation or processing. Mitotic cells were enriched by adding 100 ng/ml nocodazole for 12 h (hours 60–72 of the doxycycline treatment) and then freed from the cell culture flask surface by vigorous shaking. To enrich for early G1 cells, shaken mitotic cells were washed three times with PBS and plated onto polylysine-coated coverslips in drug-free medium, and samples were taken at specified time points. HeLa cells continuously expressing the V5-tagged p150 transgenes (Smith et al., 2014) were maintained in DMEM and supplemented with 10% FBS, 2 mM L-glutamine, and antibiotic/antimycotic solution (Life Technologies, Carlsbad, CA). RPE1-hTERT cells (a generous gift from Judith Sharp, Department of Molecular Biology, Massachusetts General Hospital) were maintained in 50:50 DMEM-F12 medium supplemented with 10% FBS, 7.5% sodium bicarbonate, 2 mM L-glutamine, and antibiotic/antimycotic solution (Life Technologies).

Depletion reagents
For lentiviral depletion (Smith et al., 2014), cells were infected at a multiplicity of infection of 7.5 with 6 µg/ml Polybrene for 72 h. Lentivirus reagents were synthesized as previously described (Campeau et al., 2009; Smith et al., 2014). Endonuclease-prepared siRNA (esiRNA) reagents were generated as previously described (Fazzio et al., 2008; Smith et al., 2014), using primers listed in Table 1 to generate double-stranded RNAs, which were then digested using RNase III. For esiRNA transfection, 500 ng of siRNAs were transfected in 1 ml Opti-MEM (Life Technologies) with 6 µl of Oligofectamine (Life Technologies). After 6.5 h, 2.5 ml of medium was added on top of the transfection cocktail, and cells were processed after 72 h. For synthetic siRNA transfection, 10 µl of 5 µM scramble (AM4611; Ambion) or Ki-67 (4392420-s8796; Ambion) siRNAs were diluted in 400 µl of Opti-MEM (Life Technologies) with 5 µl RNAiMAX (Invitrogen) and incubated at room temperature for 10 min. The siRNA cocktail was then slowly added to six-well dishes containing 800 µl of Opti-MEM, and 6 h later, 2.5 ml of appropriate medium (lacking antibiotics) was added. Note that the esiRNA directed against Ki-67 targets the Ki-67 repeat region within exon 13, whereas the synthetic siRNA targets nucleotides 559–577 (CGUCGUGUCUCAAGAUCU-Att) within exon 6.

Immunofluorescence and immuno-FISH
Cells were plated on polylysine (Sigma-Aldrich, St. Louis, MO)–coated coverslips for 24 h before manipulation. For immunofluorescence experiments, fixation and permeabilization were performed in six-well tissue culture dishes to minimize displacement of mitotic cells. After aspiration of media, cells were immediately fixed with 4%
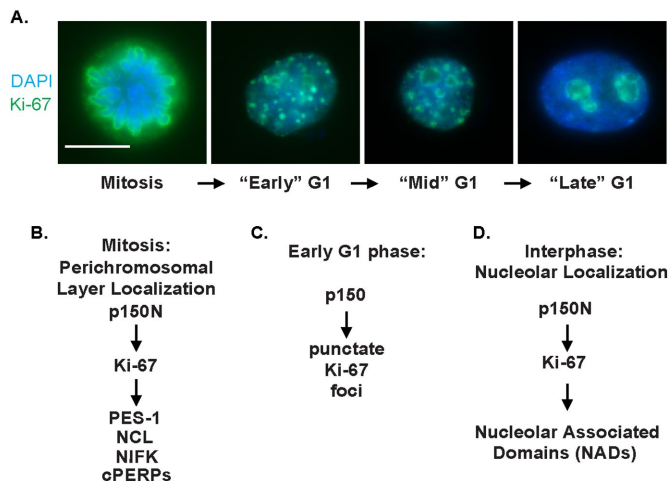
FIGURE 5: p150 regulates Ki-67 localization throughout the cell cycle: a summary of dependence relationships. (A) HeLa S3 cells were stained with DAPI to visualize DNA (blue) and antibodies against Ki-67 (green) to demonstrate Ki-67 staining patterns throughout the cell cycle. (B) Hierarchy of known proteins controlling localization to the perichromosomal layer during mitosis. Proteins dependent on Ki-67 include pescadillo (PES-1; Sobecki et al., 2016), as well as NCL, NIFK, and the cPERPs (Booth et al., 2014). (C) p150 is required for Ki-67 localization to punctate foci during early G1 (Figure 3). (D) p150N is required for localization of the Ki-67 protein and NAD loci to nucleoli during interphase (Smith et al., 2014).

PFA/PBS for 10 min at room temperature. Cells were then gently washed with ice-cold PBS and permeabilized with 0.5% Triton TX-100/PBS on ice for 5 min. Cells were then washed twice with room temperature PBS and then incubated in blocking buffer (1% BSA/PBS) for 5–30 min in a 37°C humid chamber. After blocking, cells were incubated with primary antibody in blocking buffer for 2 h in a 37°C humid chamber (Table 2). Coverslips were then transferred to

Coplin jars and washed three times with PBS on a rotating platform for 10 min at room temperature. After washing, cells were incubated with secondary antibody in blocking buffer for 1 h in a 37°C humid chamber. Coverslips were then transferred to Coplin jars and washed three times with PBS on a rotating platform for 10 min at room temperature. After the final washing step, cells were transferred to a Coplin jar containing 4′,6-diamidino-2-phenylindole (DAPI; 50 ng/ml) in PBS for 2 min. Coverslips were mounted with Vectashield (Vector Labs, Peterborough, UK), and images were taken on a Zeiss Axioplan2 microscope with a 63× objective. Corrected total cell fluorescence (CTCF) was quantified using the ImageJ "measure" feature and background subtracted from an area of the image without a cell. Images were all captured using the same exposure time across three biological replicates, and the unpaired Student's t test was used to generate p values. Immuno-FISH was performed as previously reported (Smith et al., 2014). Association percentages for each of the three biological replicates were transformed into arcsine units, and the unpaired Student's t test was used (with Welch's correction) to generate p values. $p < 0.05$ was considered statistically significant. All statistical analysis was performed using GraphPad Prism 6.

Nuclease digestion

RPE1-hTERT cells were used for these experiments rather than HeLa cells because they are sufficiently adherent during detergent permeabilization/nuclease digestion/high-salt extraction. Nuclease digestion was performed as previously reported (Sheval and Polyakov, 2008), with some modification. Cells were plated onto polylysine-coated coverslips in six-well dishes at least 24 h before manipulation, and all subsequent steps were performed with solutions containing 100 μM phenylmethylsulfonyl fluoride. Cells were washed once with digestion buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM $CuSO_4$) and then incubated in 1% Triton TX-100/digestion buffer for 10 min at 4°C. Cells were then gently washed with digestion buffer and incubated in 100 μg/ml DNase I/digestion buffer, 100 μg/ml RNase A/digestion buffer, or

Target	Forward primer	Reverse primer
Luciferase	gcgtaatacgactcactataggAACAATTGCTTTTACAGATGC	gcgtaatacgactcactataggAGGCAGACCAGTAGATCC
NCL	gcgtaatacgactcactataggGCGACGAAGATGATGAAGATGA	gcgtaatacgactcactataggGTGAGTTCCAACGCTTTCTCC
Ki-67	gcgtaatacgactcactataggGTGCTGCCGGTTAAGTTCTCT	gcgtaatacgactcactataggGCTCCAACAAGCACAAAGCAA

TABLE 1: esiRNAs.

Epitope	Species	Manufacturer or provider	Catalog number	Immunofluorescence dilution	Western blot dilution
Fibrillarin	Rabbit	Abcam, Cambridge, UK	Ab5821	1:500	1:2000
H3S28ph	Mouse (clone HTA28)	Sigma-Aldrich	H9908	1:100	
Ki-67	Rabbit	Abcam	Ab66155	1:500	1:2000
NCL	Mouse (clone 364-5)	Abcam	Ab136649	1:250	1:1000
p150	Rabbit	Campeau et al. (2009)			1:2000
p150	Mouse (ss1)	Smith and Stillman (1991)		1:500	
p150	Mouse (ss48)	Smith and Stillman (1991)		1:500	
Phospho–Ki-67	Rabbit	Takagi et al. (2014)			1:5000
V5	Mouse	Thermo	R960-25	1:1000	1:2000

TABLE 2: Antibodies.

mock digested ("untreated") in digestion buffer in a 37°C humid chamber for 30 min. Proteins were then extracted by incubating for 10 min in extraction buffer (2 M NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 20 mM Tris-HCl, pH 7.6) at 4°C. Cells on coverslips were immediately fixed as described, or RNA samples were processed using TRIzol (Invitrogen). Briefly, 1 ml of TRIzol was added directly to the plate, and cells were homogenized by pipetting. After incubation for 5 min at room temperature, samples were stored at –80°C until further processing. After thawing, 200 µl of chloroform was added, and samples were shaken vigorously for 15 s before incubation at room temperature for 2 min. Samples were then centrifuged at 13,000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, and one volume of isopropanol was added and mixed well by vigorous shaking. Samples were then incubated at room temperature for 10 min before centrifugation at 13,000 × g for 15 min at 4°C. Samples were then washed with 75% ethanol and resuspended with nuclease-free water. Ten percent of recovered samples were run on a 1% agarose gel to compare RNA digestion efficiency.

Early G1 cells were selected by choosing pairs of cells that appeared significantly smaller than surrounding cells and featured hundreds of Ki-67 foci (Isola *et al.*, 1990; du Manoir *et al.*, 1991; Croft *et al.*, 1999). Line scans were performed using the RGB Profiles Tool plug-in for ImageJ.

Western blotting

A 15-µg amount of protein was loaded and run through either Tris-HCl polyacrylamide gradient gel (20–5%) for asynchronous samples or a NuPAGE Novex 3–8% Tris-Acetate Protein Gel (Thermo-Fisher Scientific, Waltham, MA) for mitotically arrested samples. Protein samples were then transferred to a polyvinylidene fluoride membrane at 20 V for 17 h at 4°C to maximize the transfer of high–molecular weight Ki-67. Chemiluminescence was acquired using the Bio-Rad ChemiDoc system and quantified using Bio-Rad Image Lab V 5.2.1.

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