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FBW7 Loss Promotes Chromosomal Instability and Tumorigenesis via Cyclin E1/CDK2-Mediated Phosphorylation of CENP-A

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Abstract

The centromere regulates proper chromosome segregation, and its dysfunction is implicated in chromosomal instability (CIN). However, relatively little is known about how centromere dysfunction occurs in cancer. Here, we define the consequences of phosphorylation by cyclin E1/CDK2 on a conserved Ser18 residue of centromere-associated protein CENP-A, an essential histone H3 variant that specifies centromere identity. Ser18 hyperphosphorylation in cells occurred upon

loss of FBW7, a tumor suppressor whose inactivation leads to CIN. This event on CENP-A reduced its centromeric localization, increased CIN, and promoted anchorage-independent growth and xenograft tumor formation. Overall, our results revealed a pathway that cyclin E1/CDK2 activation coupled with FBW7 loss promotes CIN and tumor progression via CENP-A-mediated centromere dysfunction. *Cancer Res*; 77(18); 4881–93. ©2017 AACR.

Introduction

FBW7 is a well-established tumor suppressor (1). It belongs to the F-box family of proteins that are an essential component of the SCF (SKP1-CUL1-F-box protein) type of E3 ubiquitin ligase complex, which targets substrates for polyubiquitination followed by proteasome-mediated degradation (2). F-box proteins determine substrate specificity for the SCF ligase complex. There are many important characterized FBW7 E3 ligase substrates, including cyclin E1 (3). Loss of FBW7 in cancer leads to aberrant accumulation of substrates, accounting for many tumor pheno-

types observed in cell lines, xenograft or genetic mouse models, and human patients (3).

Chromosome instability (CIN) is a cancer hallmark that contributes to cancer progression, tumor heterogeneity, and drug resistance (4, 5). Notably, FBW7 depletion induces CIN in colon cancer cells, including mitotic defects, which can be rescued by codepletion of cyclin E1 (6). Cyclin E1/CDK2 kinase activity peaks at the G₁-S cell-cycle phase and is required for proper cell-cycle progression into the S-phase (7). However, the underlying molecular mechanism by which cyclin E1 contributes to CIN remains elusive. Chromosome stability requires the centromere, which is the specialized chromatin locus where the kinetochore is built. The centromere is enriched for centromere protein A (CENP-A), an essential histone H3 variant that serves as a key epigenetic mark for centromere identity and propagation (8). CENP-A depletion displaces the downstream components from centromeres and kinetochores, resulting in chromosome missegregation (9), and CENP-A mislocalization to noncentromeric chromatin can lead to ectopic kinetochore and fragmented chromosomes (10). Therefore, CENP-A must be tightly regulated to ensure proper centromere functions. Clinical evidence strongly correlates centromere gene misregulation with CIN and poor patient prognosis for several human cancer types (11, 12). However, the roles and mechanisms of centromere misregulation are poorly understood in the context of cancer progression.

CENP-A must be replenished in each cell division, and chromatin assembly at centromeres requires a dedicated pathway (13). Newly synthesized CENP-A protein binds to its chaperone and assembly factor Holiday junction recognition protein (HJURP), which transiently localizes to centromeres at the time of new CENP-A incorporation, from late telophase to early G₁ phase in part via MIS18 (14–17). Cell-cycle kinases tightly regulate CENP-A deposition in human cells, mostly based on data

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from cancer cell lines (18, 19). For example, phosphorylation of M18BP1 and HJURP by CDK1/2 prevents nucleosome assembly in S and G₂ phases, and inhibition of CDK1/2 activity is required for CENP-A loading (18, 19). Moreover, phosphorylation of CENP-A at Ser68 mediated by cyclin B/CDK1 might also be important for proper CENP-A localization despite some debates (20–22). Finally, phosphorylation of CENP-A N-terminus at Ser16 and Ser18 residues has been implicated in chromosome segregation (23). However, the exact roles and pathways of CENP-A misregulation in cancer progression are poorly understood.

In this study, we show that *FBW7* loss significantly compromises CENP-A deposition and reduces CENP-A levels at centromeres in human colon and breast cancer cell lines. *FBW7* loss promotes excessive cyclin E1/CDK2-mediated CENP-A phosphorylation at the serine 18 (Ser18) residue in the N terminal tail. We show that human cyclin E1/CDK2 is a bona fide CENP-A Ser18 kinase using *in vitro* and *in vivo* assays. Persistent CENP-A Ser18 phosphorylation caused by *FBW7* loss results in increased frequencies of lagging chromosomes, chromosomal bridges, and micronuclei formation, which could be rescued by codepletion of cyclin E1. In addition, the phosphor-mimetic CENP-A S18D mutant phenocopies *FBW7* loss and promotes xenograft tumor growth. We suggest a novel mechanism by which *FBW7* loss contributes to CIN and tumorigenesis.

Materials and Methods

Cell culture

Wild-type (WT) and DLD1 *FBW7*^{−/−} cell lines were kindly provided by Dr. Bert Volgestein (Johns Hopkins University, Baltimore, MD) in 2004 and were cultured in RPMI supplemented with 10% FBS. MCF10A cells were cultured in MEM (Lonza) containing 52 μg/mL bovine pituitary extract, 500 ng/mL hydrocortisone, 10 ng/mL hEGF, 5 μg/mL insulin (MEGM Bullet Kit, Lonza Corporation), and 100 ng/mL cholera toxin (Sigma-Aldrich). MDA-MB-453 cells were maintained in DMEM with 10% FBS. These were obtained from ATCC from 2013 to 2016. All cells were maintained at 37°C in 5% CO₂ incubator. For synchronization, cells were arrested 18 (DLD1) or 20 hours (DLD1 *FBW7*^{−/−}) in 330 nmol/L nocodazole, collected by mitotic shake-off, and released by thorough washout. FACS analysis was performed as described previously (24).

Antibodies and plasmids

Antibodies used in this study are listed in Supplementary Table S1. The phospho-CENP-A (Ser18) antibody was generated and provided as a kind gift by Cell Signaling Technology. HA-tagged WT or mutant *FBW7* and cyclin E1 were described previously (24). HA-CCNE1 R130A mutant was generated by site-directed mutagenesis and confirmed by sequencing. Full-length HA-tagged CENP-A was amplified by PCR with a 5′ primer that introduced a BamHI site and an HA tag and a 3′ primer that introduced an SalI site. The PCR product was digested with BamHI and SalI and cloned into pLenti CMV GFP vector (Addgene). Mutant CENP-A constructs were generated by site-directed mutagenesis and confirmed by sequencing.

siRNAs, lentiviral shRNA vectors, and generation of stable cell lines

Nontargeting siRNA no. 2 (D0012100220) and cyclin E1 smartpool siRNA (LQ-003213-00-0002) were obtained from

Dharmacon. Lentiviral *FBW7*, CENP-A, cyclin E1, cyclin A, CDK1, CDK2, and cyclin B shRNAs were obtained from the Broad Institute TRC shRNA library. Lentiviral shRNA virus packaging, retrovirus packaging and subsequent infections were performed as described previously (25). Target sequences are listed as below:

Ctrl shRNA: AACAGTCGCGTTTGGCGACTGG
FBW7 (#1): CCAGTCGTTAACAAGTGGAAT
FBW7 (#2): CCAGAGACTGAAACCTGTCTA
CENP-A (694): CCGAGTTACTCTCTCCCCAAA
CENP-A (695): GCCTATCTCCTCACCTTACAT
CENP-A (697): GCAGCAGAAGCATTCTAGIT
Cyclin E1 (#1): CGACATAGAGAACTGTGTCAA
Cyclin E1 (#2): CCTCCAAAGTTGCACCAAGTTT
Cyclin A (#1): AAGGCAGCGCCCGTCCAAACA
Cyclin A (#2): AACTACATTG ATAGGTTCTCG
CDK2 (#1): GCCTTCCTACACGTTAGATTT
CDK2 (#2): CCGAGAGATCTCTCTGCTTAA
CDK1 (#1): GGATGTGCTTATGCAGGAT
CDK1 (#2): GGGGTTCTAGTACTGCAA
Cyclin B: GTCAGTGAACAACCTGCAGG

CENP-A-SNAP loading assays

DLD1 cells stably expressing CENP-A-SNAP proteins were synchronized to G1 using double thymidine block. Cells were released into normal media and BTP was added to quench the preexisting CENP-A-SNAP pool. After removal of BTP, cells were allowed to grow for 11 hours before addition of TMRstar to label newly synthesized CENP-A-SNAP proteins as described previously (14). BTP and TMRstar were purchased from New England Biolabs.

Chromatin extraction and Western blot analysis

Whole-cell lysate extraction and immunoprecipitation experiments were performed as described previously (25). Chromatin-bound fractions were extracted similarly as described previously (26). Specifically, cells were collected at 95% confluency for chromatin-bound fraction extraction. Cell pellets containing nuclei were resuspended with 0.4 N H₂SO₄, followed by thorough resuspension and centrifugation. TCA solution (final concentration 33%) was added to the cleared supernatant to precipitate chromatin-bound fraction, followed by resuspension in EBC lysis buffer as described previously (25).

To determine the specificity of the pS18 CENP-A antibody raised in this study, HeLa cell nuclei were harvested and digested with MNase before chromatin extraction with salt as described previously (15). CENP-A chromatin was immunoprecipitated using either anti-HA mAb after transfection of HA-tagged CENP-A constructs, or rabbit anti-total CENP-A polyclonal antibody for endogenous CENP-A proteins before Western blotting.

Immunofluorescence and image quantification

Immunofluorescence was conducted using methods described previously (27). Human mitotic chromosomes were prepared by cytospin as described previously (28). Integrated fluorescence intensity measurements were performed by using the methods described previously (29). Primary antibodies were listed in Supplementary Table S1. Images were deconvolved and projected using SoftWoRx (GE Healthcare) for presentation. Raw images

were analyzed using CRaQ v1.12 in Fiji for centromeric foci analysis as described previously (30).

GST protein purification and GST pull down

The GST-Skp2 was generated by fusing the first 90 residues of mouse Skp2 in frame with the GST tag as described previously (31). GST plasmids were transformed with BL21 competent cells, followed by purification and pull down experiments as described previously (25).

In vitro kinase assays and mass spectrometry analysis

Active Cdk2/cyclin kinases were purchased from Millipore, and the protocol was followed as described previously (32). Following *in vitro* kinase assay, mass spectrometry analysis was performed similarly as described previously (25).

CRISPR-Cas9 for CENP-A knockins

GeneArt seamless (Life Technologies) or G-blocks (IDT) were used to generate either the WT or phospho-mimic mutant of CENP-A (CENP-A S18D). See Supplementary Methods for amino-acid sequences that were designed for CRISPR Knockin. The human codon-optimized Cas9 (Addgene #52961) plasmid was obtained from Addgene. sgRNAs and right arm (CENP-A WT and CENP-A S18D), left arm, and insertion tag sequence were designed and constructed by using the method described previously (33). Potential off-target effects of sgRNA candidates were analyzed by the online tool CRISPR Design developed by Zhang's laboratory (<http://crispr.mit.edu/>), and the sgRNA sequences with fewer off-target sites in human genome were selected for further analysis. For donor vectors, the requisite 15-bp end terminal homology between adjacent fragments or vector were designed and generated by IDT.

Synthesized double-stranded left arm, right, SNAP tag as well as linearized donor vector was ligated using the GeneArt seamless enzyme (Life Technologies). Following ligation and transformation, DNA was prepared and sequenced to confirm the appropriate ligation. DLD1 cells were then transfected with FBW7 sgRNAs and ligated knockin vector with the SNAP tag followed by puromycin selection. Puromycin-selected cells were divided into single-cell culture, followed by cell line expansion, genomic DNA extraction, PCR, and sequence validation to confirm successful knockins.

FBW7 sgRNA sequence:

5'-CACCGCACCTCTGCGCGGTGTCATGG-3'

5'-AAACTGACACGCCGAGAGGGTGCC-3'

Soft agar assays and xenograft tumor growth analysis

Soft agar assays were performed as described previously (34). For xenograft experiments, 5×10^5 cells with 10% Matrigel were injected into the hind flank of 6-week-old female NOD/SCID gamma mice. Tumor sizes were measured every 3 to 4 days after initial tumor growth using a caliper. The tumor volume was then determined using the formula: $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter of the measured growth. After 42 days, mice were euthanized, and tumors were harvested and weighed. All animal experiments were complied with NIH guidelines and were approved by the University of North Carolina at Chapel Hill Animal Care and Use Committee.

Statistical analysis

Results were reported as means $\pm$ SEM from three independent experiments where applicable. The imaging data were analyzed by GraphPad Prism7 (GraphPad software) unless otherwise indicated. Wilcoxon rank sum test, two-tailed unpaired Student *t* test, Mann-Whitney *U* test, and Fisher exact test were used to analyze statistical significance as indicated. *P* value <0.05 was considered statistically significant.

Results

FBW7 loss leads to reduced CENP-A localization at the centromere

To determine whether FBW7 loss in cancer cells is accompanied by any centromere defects, we first examined CENP-A protein levels in chromatin fractions from isogenic DLD1 colon cancer cell lines with and without FBW7 knockout (24). Using a validated CENP-A antibody (Supplementary Fig. S1A), we found that FBW7^{-/-} cells displayed decreased CENP-A levels in the chromatin fraction compared with FBW7^{+/+}, whereas total CENP-A in whole-cell lysates was unchanged (Fig. 1A). Interestingly, CENP-A decreases in the chromatin fraction was accompanied by a simultaneous increase in CENP-A levels in the soluble fraction (Fig. 1B), accounting for the unchanged CENP-A levels in whole-cell lysates. Importantly, ectopic reintroduction of FBW7 to DLD1 FBW7^{-/-} cells decreased the level of the canonical FBW7 E3 ligase substrate cyclin E1 and rescued CENP-A protein levels in the chromatin fraction, supporting a specific role for FBW7 in regulating CENP-A levels in chromatin (Fig. 1A; Supplementary Fig. S1B). However, an FBW7 R465H hotspot mutation identified in various cancer types from patients, which is known to cause complete loss of FBW7 E3 ligase activity (35), failed to restore cyclin E1 and chromatin-bound CENP-A protein levels (Supplementary Fig. S1B).

We then compared centromeric CENP-A levels between DLD1 FBW7-null and WT cells by immunofluorescence (IF) analysis, using CENP-C staining to identify kinetochores. Loss of FBW7 results in a marked reduction in CENP-A signal at centromeres compared with WT cells, in both metaphase and interphase ($P < 0.0001$, Student *t* test; Fig. 1C and D). FBW7 depletion using two independent shRNAs (#1 and #2) in the MDA-MB-453 breast cancer cell line and an immortalized breast epithelial cell line MCF-10A also showed similar results (Fig. 1E; Supplementary Fig. S1C). There is no significant effect on total CENP-A levels, suggesting that CENP-A stability is not impacted (Fig. 1E; Supplementary Fig. S1C). Notably, high levels of ectopic overexpression of FBW7 also caused a moderate drop in total CENP-A via an unknown mechanism (Fig. 1A).

Decreased CENP-A signals at centromeres could result from the loss of preexisting CENP-A or defective new CENP-A nucleosome assembly, which we addressed using a quench-chase-pulse SNAP-tagged CENP-A assay (Fig. 1F; ref. 14). We first generated DLD1 FBW7^{+/+} and FBW7^{-/-} clones that stably express CENP-A-SNAP with mild overexpression confirmed by Western blotting and IF, or TMRstar labeling without BTP quenching (Supplementary Fig. S1D and S1E). To determine whether new CENP-A loading is impacted by FBW7 loss, the preexisting CENP-A-SNAP pool was quenched with BTP after a double thymidine block, and nascent protein was labeled with TMRstar for localization in the next G₁ phase (14), in FBW7 WT and knockout cells, respectively (Fig. 1F). Notably, FBW7^{-/-} cells displayed a significant decrease of

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Figure 1.

FBW7 loss leads to defects in CENP-A centromeric localization. **A** and **B**, Immunoblot of CENP-A using whole-cell extracts, chromatin-bound and soluble fractions prepared from DLD1 cells (*FBW7*^{+/+}), *FBW7* knockout (*FBW7*^{-/-}) or *FBW7*^{-/-} cells with reintroduction of exogenous *FBW7*. **C** and **D**, Representative images (**C**) and quantification (**D**) of *FBW7*^{+/+} or *FBW7*^{-/-} DLD1 cells stained for CENP-A (green) or CENP-C (red). Cells were costained with anti-tubulin antibody (blue) to identify mitotic cells. Images are maximum-intensity projections of z stacks collected at 0.5 μm/L steps. Scale bar, 5 μm. Results were statistically significant using unpaired Student *t* test. Data are shown as mean ± SEM. **E**, Immunoblot of lysates from MDA-MB-453 infected with lentiviruses encoding *FBW7* shRNA (#1 and #2) or control shRNA (Ctrl). **F**, Experimental scheme for CENP-A loading assay in DLD1 CENP-A-SNAP cells. **G**, DLD1 *FBW7*^{+/+} or *FBW7*^{-/-} cells expressing CENP-A-SNAP were quenched with BTP, followed by immediate TMRstar (green) incubation with no chase to assess blocking efficiency (top two panels), or quenched with BTP, then allowed 11 hours chase time (bottom two panels) for new protein synthesis before labeling new CENP-A-SNAP with TMRstar. Anti-SNAP antibody was used to stain all CENP-A-SNAP (red). DNA was stained by DAPI (blue). Scale bar, 5 μm. **H**, Box plots of CENP-A-SNAP loading at centromeres using the ratio of nascent (TMRstar) over total CENP-A-SNAP signals (anti-SNAP antibody). Mann-Whitney *U* test was used to determine statistical significance.

1.91-fold in TMRStar staining compared with *FBW7*^{+/+} cells ($P < 0.0001$, Wilcoxon rank sum test; Fig. 1G and H).

The cyclin E1/CDK2 kinase complex mediates the CENP-A reduction at centromeres after *FBW7* loss

Several studies suggested that mitotic cyclins and CDK1/2 kinase activity are inhibitory for ectopic CENP-A loading by mediating phosphorylation of M18BP1, HJURP, and the Ser68 residue of CENP-A itself (18–20). Cyclin E1 forms a functional kinase complex with CDK2 at the G₁-S boundary to regulate cell-cycle progression into the S-phase (36). *FBW7* loss induces accumulation of cyclin E1 (Fig. 1A), raising the possibility that *FBW7* impacts CENP-A localization through cyclin E1. To test this hypothesis, we determined the impact of cyclin E1 depletion by siRNAs on CENP-A in *FBW7*^{-/-} cells after fractionation (Fig. 2A). First, we observed that depletion of cyclin E1 in *FBW7*^{-/-} cells increased CENP-A levels in the chromatin fraction with a concomitant decrease in soluble fraction (Fig. 2A). Consistently, *cyclin E1* depletion increased centromeric CENP-A signals in both metaphase and interphase cells by IF and image quantification ($P < 0.0001$, Student *t* test; Fig. 2B and C). In a

complementary set of experiments, depletion of cyclin E1 by two independent shRNAs (#1 and #2) in *FBW7*^{-/-} cells, to levels comparable with *FBW7*^{+/+} cells, consistently restored CENP-A protein levels in the chromatin fraction (Fig. 2D). It is worth noting that chromatin-bound CENP-A levels in *FBW7*^{-/-} cells with *cyclin E1* loss were higher than *FBW7*^{+/+} cells, which could be due to a combinatorial effect of *FBW7* loss and *cyclin E1* depletion. Similarly, depletion of *CDK2* by two different shRNAs also partially restored CENP-A levels at centromeres in *FBW7* knockout cells in both Western blotting and IF experiments (Supplementary Fig. S2A–S2C). Previous studies showed that both cyclin E1 and cyclin A bind CDK2 and regulate cell-cycle progression (36, 37). However, unlike cyclin E1, *cyclin A* depletion failed to restore CENP-A in chromatin and at centromeres in *FBW7*^{-/-} cells (Supplementary Fig. S2D–S2F). In addition to CENP-A, we also examined localization of the centromere and kinetochore components CENP-B and HEC1 by IF and image quantification. Interestingly, *FBW7*^{-/-} cells also displayed decreased levels for CENP-B at centromeres, and to a lesser extent for the outer kinetochore protein HEC1, and these phenotypes are also ameliorated by *cyclin E1* depletion ($P < 0.0001$ for all comparisons, Student *t* test; Fig. 2E–H).

Figure 2.

Cyclin E1/CDK2 kinase complex mediates the reduction of centromeric CENP-A localization after *FBW7* loss. **A**, Immunoblot analysis of lysates prepared from DLD1 *FBW7*^{-/-} cells transfected with cyclin E1 smartpool siRNA or control siRNA (Ctrl). **B** and **C**, Representative images (**B**) and quantifications (**C**) of DLD1 *FBW7*^{-/-} cells transfected with cyclin E1 siRNA or control siRNA stained with CENP-A (green) or CENP-C (red). DNA was stained by DAPI (blue). Scale bar, 5 μm. **D**, Immunoblot analysis of lysates prepared from DLD1 *FBW7*^{+/+} or *FBW7*^{-/-} cells infected with lentivirus encoding either cyclin E1 shRNAs (#1 and #2) or control shRNA (Ctrl). **E-H**, Representative images of mitotic cells after cytospin (left, **E**) and interphase cells by whole mount (right, **E**), and quantifications (**F-H**) of DLD1 *FBW7*^{-/-} cells infected with the lentivirus encoding either control shRNA (Ctrl) or cyclin E1 shRNA (#1 and #2) stained for CENP-B (red) and HEC1 (green). DNA was stained by DAPI (blue). Scale bar, 5 μm. Data were shown as mean ± SEM.

Cyclin E1/CDK2 kinase complex phosphorylates CENP-A at Ser18 *in vitro*

To determine how cyclin E1/CDK2 affects centromeric CENP-A localization, we tested the hypothesis that cyclin E1/CDK2 directly phosphorylates CENP-A. We identified a canonical CDK substrate motif (S/TP) at the N-terminus of CENP-A at Ser18, which is well conserved across mammalian species (Fig. 3A). Previous research showed that CENP-A Ser18 is phosphorylated in HeLa and U2OS cells (23, 38), which we confirmed by mass spectrometry (data not shown). We then determined if the cyclin E1/CDK2 kinase can phosphorylate GST-tagged full-length CENP-A *in vitro*. Incubation with recombinant cyclin E1/CDK2 kinase complex led to efficient CENP-A phosphorylation by radiography after separating proteins by SDS-PAGE, at a comparable level with a known cyclin E1/CDK2 substrate Skp2 (Fig. 3B; ref. 24). To determine whether cyclin E1/CDK2 phosphorylates CENP-A specifically at Ser18 residue or other adjacent sites, we mutated CENP-A at the Ser16, Ser18, Thr20, and Thr22 residues to alanines, either indi-

vidually or together at all four residues (termed "4A"). We observed that only the Ser18 single mutation and 4A mutation abolished most CENP-A phosphorylation mediated by cyclin E1/CDK2 (Fig. 3C). Mass spectrometry analysis of CENP-A proteins showed Ser18 as the major phosphorylation site after *in vitro* kinase assays (Fig. 3D).

Next, we raised an antibody that specifically recognizes CENP-A phosphorylated at Ser18 (pS18-CENP-A). This antibody only recognizes CENP-A peptides with pS18 single or pS16/pS18 double phosphorylation, but not the unmodified or pS16 single phosphorylation peptide on dot plots (Fig. 3E; Supplementary Fig. S3A). Moreover, CENP-A depletion by siRNA effectively reduced antibody signals by Western blotting using immunoprecipitated endogenous CENP-A (Supplementary Fig. S3B). In addition, this antibody only recognized immunoprecipitated HA-tagged CENP-A WT proteins by Western blotting but not after the Ser18 residue was mutated to an alanine (S18A; Supplementary Fig. S3C). Finally, *FBW7*^{-/-} cells display significantly higher levels

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Figure 3.

The cyclin E1/CDK2 kinase complex phosphorylates CENP-A at Ser18 *in vitro*. **A**, N-terminal CENP-A amino acid sequence alignments in selected species. **B** and **C**, Autoradiography (top) and SDS-PAGE Coomassie Blue staining (bottom) after *in vitro* cyclin E1/CDK2 kinase assay using recombinant GST-CENP-A constructs and γ -³²P-ATP. Arrow, GST-tagged cyclin E1. His-tagged CDK2 is expected to be masked by GST due to similar molecular weight. **D**, LC/MS-MS data for the excised CENP-A band showing CENP-A peptides phosphorylated at Ser18. **E**, Dot blot analysis of pS18 CENP-A and biotin antibodies for biotinylated CENP-A peptides (AA12-27) that are either WT (non-phospho) or phosphorylated on Ser18 (phospho S18) residue. **F**, Immunoblots of pS18-CENP-A in chromatin-bound extracts from DLD1 FBW7^{+/+} or FBW7^{-/-} treated with either CIP phosphatase (330 nmol/L) or buffer only (Ctrl) at 37°C for 1 hour.

of pS18-CENP-A compared with FBW7^{+/+} cells and phosphatase treatment completely abrogated pS18-CENP-A signals in both cell lines (Fig. 3F), demonstrating the specificity of the antibody against phosphorylated CENP-A at Ser18.

Cyclin E1/CDK2 kinase regulates CENP-A phosphorylation at Ser18 *in vivo*

We then tested whether increased pS18-CENP-A levels in FBW7^{-/-} cells require cyclin E1, by comparing CENP-A Ser18 phosphorylation levels from telophase to early G₁ in FBW7^{-/-} cells. Cells were arrested at metaphase in nocodazole and released into normal media for 3 hours (Supplementary Fig. S4A). Cyclin E1 depletion in FBW7-null cells resulted in significantly reduced pS18-CENP-A and increased total CENP-A levels in the chromatin fraction at each time point from telophase to early G₁, while total CENP-A levels in whole-cell extracts remained constant (Fig. 4A). Moreover, quantification of IF experiments showed that pS18-CENP-A signals at centromeres are higher in both interphase and mitotic cells in FBW7^{-/-} cells compared with FBW7^{+/+} cells, but decreased significantly after cyclin E1 depletion (Supplementary Fig. S4B and S4C).

We then investigated whether overexpression of cyclin E1 enhances pS18-CENP-A levels in FBW7^{+/+} cells. We first arrested

DLD1 FBW7^{+/+} cells in early S-phase or metaphase with thymidine and nocodazole, respectively. CENP-A Ser18 phosphorylation in chromatin was highly enriched in thymidine-treated cells, coinciding with the peak of cyclin E1 levels, but significantly diminished in cells treated with nocodazole (Supplementary Fig. S4D). Similarly, in DLD1 FBW7^{+/+} cells, pS18-CENP-A signals at centromeres were low in G₂ and most of mitosis, but started to increase in telophase cells by IF experiments (Supplementary Fig. S4E and S4F).

Moreover, DLD1 FBW7^{+/+} cells with or without overexpression of HA-cyclin E1 (HA-CycE1) were synchronized by nocodazole and released into normal media, respectively (24). After release from metaphase arrest, DLD1 cells entered into telophase/G₁ within 3 hours (Supplementary Fig. S4G). Cyclin E1 overexpression in DLD1 FBW7^{+/+} cells elevated levels of CENP-A Ser18 phosphorylation and simultaneously decreased chromatin-bound CENP-A (Fig. 4B). IF analysis confirmed that centromeric CENP-A levels were reduced to about 50% in cyclin E1-overexpressing cells compared with control cells ($P < 0.0001$, Student *t* test; Fig. 4C–F). To determine whether the reduction in total centromeric CENP-A upon cyclin E1 overexpression in DLD1 FBW7^{+/+} cells requires a functional cyclin E1/CDK2 kinase, we infected DLD1 cells with either WT cyclin E1 or a cyclin E1 R130A

Figure 4.

Cyclin E1/CDK2 kinase regulates CENP-A phosphorylation at Ser18 *in vivo*. **A** and **B**, Immunoblot analysis of lysates prepared from DLD1 *FBW7*^{-/-} cells infected with cyclin E1 shRNAs (**A**; #1 and #2), or *FBW7*^{+/+} cells infected with an HA-cyclin E1 (**B**; HA-CycE1) lentivirus. **C**, Immunoblot analysis of whole-cell extracts from DLD1 cells infected with the lentivirus encoding WT HA-cyclin E1 (WT), HA-cyclin E1 R130A mutant (R130A), or empty vector control (Ctrl). **D**, Representative CENP-A (green) immunofluorescence images of both mitotic and interphase cells using cell lines as in **C**. Centromeres were identified by CENP-C (red). Mitotic cells were identified by tubulin (blue). **E** and **F**, Quantitation of centromeric CENP-A signal intensities using the indicated DLD1 cells in mitosis (**E**) and interphase (**F**). *P* values were calculated by unpaired Student *t* test. Data are shown as mean ± SEM. **G**, Immunoblot analysis of pS18-, pS68-, and total CENP-A in whole-cell extracts from HeLa cells transfected with the indicated siRNAs.

mutant that cannot bind CDK2 (Fig. 4C; ref. 39). IF analysis showed that cells expressing WT cyclin E1 displayed decreased total CENP-A at centromeres compared with the control cells, whereas the cyclin E1 R130A mutant had no effect on centromeric CENP-A levels (Fig. 4D–F).

To extend the observations in DLD1 cells, we also depleted *cyclin E1* or *CDK2* by two independent shRNAs in HeLa cells, respectively, and confirmed the decrease of pS18-CENP-A (Fig. 4G). A recent study suggested that cyclin B1/CDK1 phosphorylates CENP-A at Ser68 (20). Depletion of *cyclin E1* or *CDK2* by shRNAs had no impact on CENP-A Ser68 phosphorylation levels by Western blotting (Fig. 4G). Conversely, depletion of *cyclin B1* or *CDK1* only specifically reduced levels of pS68-CENP-A, with no impact on pS18-CENP-A (Fig. 4G). Thus, CENP-A Ser18 and Ser68 phosphorylation in cells are regulated by cyclin E1/CDK2 and cyclin B/CDK1 kinases, respectively.

CENP-A Ser18 phosphorylation inhibits CENP-A localization to centromeres

Our data so far strongly suggested a connection between CENP-A Ser18 phosphorylation and reduced centromeric CENP-A localization. To test this hypothesis directly, we first overexpressed shRNA-resistant exogenous CENP-A WT or a phospho-mimetic S18D mutant in *FBW7*^{+/+} cells after depleting endogenous CENP-A (data not shown). Indeed, ectopically expressed WT CENP-A localized to centromeres labeled by the anti-centromere antibody in *FBW7*-proficient cells, whereas the S18D CENP-A mutant strongly decreased at centromeres by IF (Fig. 5A). Consistently, the CENP-A S18D-mutant protein displayed decreased levels in chromatin compared with WT CENP-A by Western blotting (Fig. 5B). Our cell fractionation experiments showed that a significant fraction of S18D CENP-A localized to the soluble fraction, while the WT CENP-A predominantly localized to chromatin (Fig. 5C). Conversely, we depleted endogenous CENP-A by shRNA followed by expression of WT or phosphorylation-deficient S18A CENP-A mutant protein from shRNA-resistant constructs in *FBW7*^{-/-} cells. Notably, S18A CENP-A-expressing cells displayed increased levels of chromatin-bound CENP-A protein and centromere localization compared with cells expressing WT CENP-A (Fig. 5D and E).

Deposition of CENP-A at centromeres is mediated by the HJURP chaperone (15, 16). We aimed to determine whether S18 phosphorylation affects CENP-A binding with HJURP. Our results showed that recombinant GST-CENP-A S18D mutant pulled down much less HJURP than GST-CENP-A WT (Fig. 5F), while both constructs pulled down similar amount of a different CENP-A-binding protein CENP-B (Fig. 5F). In a complementary set of experiments, recombinant GST-HJURP pulled down less CENP-A S18D mutant than CENP-A WT (Fig. 5G). Furthermore, we analyzed recruitment of CENP-A to a LacO (Lac operon) array by an HJURP-LacI fusion protein *in vivo* (40). Chinese hamster ovary DG44 cells (Named as A03_1) containing LacO arrays at a single noncentromeric locus (Supplementary Fig. S5A; ref. 20) were cotransfected with mCherry-LacI-fused HJURP and EGFP-tagged WT, S18D or S18A mutant CENP-A, respectively. For EGFP-tagged WT or S18A CENP-A, we observed EGFP signals predominately at the LacO arrays as indicated by colocalization with mCherry-LacI-HJURP. However, a significantly higher proportion of cells expressing CENP-A S18D showed more diffuse EGFP signals at regions outside of the LacO arrays marked by mCherry-LacI-HJURP (Supplementary Fig. S5B and S5C),

implying that HJURP cannot efficiently recruit CENP-A S18D-mutant proteins.

CENP-A Ser18 phosphorylation causes mitotic defects and CIN

Next, we examined the effect of the S18D mutant on mitotic defects, including lagging chromosomes and chromosome bridges. Stable expression of S18D CENP-A in DLD1 *FBW7*^{+/+} cells led to increased bridging or lagging chromosomes compared with DLD1 *FBW7*^{+/+} control ($P < 0.01$, Fisher exact test; Fig. 6A and B). Conversely, stable expression of a CENP-A S18A phospho-deficient mutant, or stable cyclin E1 depletion by shRNA in DLD1 *FBW7*^{-/-} cells, significantly suppressed the frequencies of chromosome bridging and lagging phenotypes in *FBW7*^{-/-} cells ($P < 0.01$, Fisher exact test; Fig. 6A and B). To further investigate the role of Ser18 phosphorylation in centromere function in other cell lines, we also stably expressed either CENP-A WT or S18D phospho-mimetic mutant proteins tagged with EGFP at low levels in HeLa cells (Supplementary Fig. S6A). IF analysis showed that S18D EGFP-CENP-A had significantly weaker centromeric CENP-A localization than WT EGFP-CENP-A (Supplementary Fig. S6B and S6C). HeLa cells expressing the S18D mutant also displayed significantly increased mitotic defects compared with the WT control (Supplementary Fig. S6B and S6C).

Micronucleus formation is a marker for increased CIN and cancer risk (6, 41) and can be generated by lagging or bridging chromosomes during cell division (42, 43). Staining for LAP2, a lamin- and chromatin-binding nuclear protein (44, 45), revealed that DLD1 *FBW7*^{-/-} cells display increased micronucleus frequencies compared with *FBW7*^{+/+} cells and that this phenotype is eliminated by cyclin E1 depletion ($P < 0.01$, Fisher exact test; Fig. 6C and D). Strikingly, expression of phospho-mimetic S18D CENP-A in DLD1 *FBW7*^{+/+} cells significantly increased micronucleus frequencies compared with *FBW7*^{+/+} controls ($P < 0.01$, Fisher exact test), whereas expression of phospho-deficient S18A CENP-A in DLD1 *FBW7*^{-/-} cells (DLD1 *FBW7*^{-/-} S18A CENP-A) significantly reduced micronucleus formation in DLD1 *FBW7*^{-/-} ($P < 0.05$, Fisher exact test; Fig. 6E and F). Consistent with published results where loss of CENP-A by shRNA (DLD1 *FBW7*^{+/+} shCENP-A) also resulted in elevated micronucleus frequencies (46) compared with controls (DLD1 *FBW7*^{+/+}), the micronucleus phenotype induced by CENP-A shRNA was only suppressed by expression of shRNA-resistant WT CENP-A ($P < 0.01$, Fisher exact test) but not by S18D CENP-A ($P > 0.05$, Fisher exact test; Supplementary Fig. S6D and S6E). These results suggest that reduced centromeric CENP-A localization due to Ser18 phosphorylation contributes to elevated levels of chromosome missegregation and micronucleus formation.

CENP-A Ser18 phosphorylation promotes tumor progression

High levels of CIN predict poor prognosis in cancer patients, and *FBW7* loss or cyclin E1 overexpression correlates with more aggressive tumor formation (47). To determine the clinical relevance of the findings, we first examined breast cancer samples for any potential correlation between cyclin E1, CENP-A pS18, and chromatin retention of CENP-A. We detected a correlation between high cyclin E1 protein levels, increased chromatin-bound CENP-A Ser18 phosphorylation, and decreased CENP-A levels in chromatin (Fig. 7A).

To directly determine whether CENP-A misregulation plays any role in tumor progression, we first compared anchorage-independent growth between cells expressing either WT or S18D phospho-

Figure 5.

CENP-A Ser18 phosphorylation inhibits centromeric localization of CENP-A. **A** and **E**, Representative images of cells from DLD1 FBW7^{+/+} cells (**A**) infected with a CENP-A shRNA lentivirus, followed by infection with either WT HA-CENP-A (WT), S18D, or empty vector (Ctrl), and DLD1 FBW7^{-/-} cells (**E**) infected with a CENP-A shRNA lentivirus, followed by infection with either WT HA-CENP-A (WT), S18A, or empty vector (Ctrl) stained with HA (green), anti-centromere antibody (ACA; red), and DAPI for DNA (gray). Scale bar, 5 μ m. **B** and **D**, Immunoblot analysis of whole-cell extracts (WCE) and chromatin extracts using the same stable cell lines in **A** and **E**. **C**, Immunoblot analysis of lysates from soluble and chromatin fractions using cells lines from **A**. Arrows, nonspecific bands that may be due to secondary effects caused by ectopic expression of CENP-A S18D. **F**, Immunoblot analysis of lysates from GST pull-down (GST control, GST-CENP-A WT or GST-CENP-A S18D) of 293T cells transfected with HA-tagged HJURP. CENP-B was also included for comparison. **G**, Immunoblot analysis of lysates from GST pull-down (GST control or GST-HJURP) using DLD1 FBW7^{+/+} cells infected with a CENP-A shRNA lentivirus, followed by infection with either WT HA-CENP-A (WT) or S18D.

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Figure 6.

Ser18 CENP-A phosphorylation leads to mitotic defects and chromosomal instability. **A** and **B**, Representative images (**A**) and quantification (**B**) of mitotic defects (chromatin bridges or lagging chromosomes) in DLD1 *FBW7*^{+/+} cells infected with the lentivirus encoding either CENP-A S18D or control, as well as DLD1 *FBW7*^{-/-} cells infected with the lentivirus encoding CENP-A S18A, cyclin E1 shRNAs (#1 and #2, respectively), or control. Cells were stained for ACA (red), actin (ACTA, green), and DNA by DAPI (blue). Scale bars, 5 μ m. **C-F**, Representative LAP2 antibody staining (green) and quantitation of micronuclei in various cell lines described and characterized in Figs. 2D, 5B, and D. Scale bar, 5 μ m. Over 200 cells were quantified for each cell type. Statistical significance was determined by Fisher exact test. (*, $P < 0.05$; **, $P < 0.01$).

Figure 7.

Ser18 CENP-A phosphorylation enhances tumor progression. **A**, Immunoblot analysis of CENP-A, pS18-CENP-A, and cyclin E1 using whole-cell and chromatin-bound extracts prepared from breast cancer patient samples. Histone H3 and vinculin served as loading controls. **B–D**, Immunoblot analysis of CENP-A (**B**), anchorage-independent growth assays (**C**), and quantitation of DLD1 *FBW7*^{+/+} cells with endogenously tagged SNAP-CENP-A WT or S18D mutant (**D**). Colony numbers in **D** are mean $\pm$ SEM. **E–G**, The tumor growth curves of tumor xenografts (**E**), gross tumor weight at the time of necropsy (**F**), and representative images of tumor size (**G**) using cell lines described in **B**. Tumor cells were transplanted subcutaneously into the bilateral dorsal region of female NOD/SCID mice. Results were statistically significant using unpaired Student *t* test. ***, *P* < 0.005. **H and I**, Anchorage independent growth assays (**H**) and quantitation of DLD1 *FBW7*^{-/-} cells with exogenously tagged CENP-A WT or S18A mutant (**I**). Colony numbers in **I** are mean $\pm$ SEM. **J–L**, The tumor growth curves of tumor xenografts (**J**), gross tumor weight at the time of necropsy (**K**), and representative image of tumor size (**L**) for cell lines described in Fig. 5D. Tumor cells were transplanted subcutaneously into the bilateral dorsal region of female NOD/SCID mice. Tumor volumes were measured with a caliper. Results were statistically significant using unpaired Student *t* test. ***, *P* < 0.005. **M**, A model for the misregulation of CENP-A hyperphosphorylation, reduced deposition, and chromosome instability in *FBW7*-mutant cells.

mimetic CENP-A in DLD1 *FBW7*^{+/+} cells. We used CRISPR-Cas9 to replace one endogenous CENP-A locus with either a WT or S18D mutant CENP-A containing an SNAP tag (Supplementary Fig. S7 and S7B). Notably, the CENP-A S18D mutant enhanced anchorage-independent growth and xenograft tumor growth compared with WT CENP-A (*P* < 0.001, Student *t* test; Fig. 7C–G). Conversely, ectopic expression of the phosphorylation-deficient CENP-A S18A mutant significantly decreased anchorage-independent growth and xenograft tumor growth in DLD1 *FBW7*^{-/-} cells compared with the cells expressing WT CENP-A as the control at a similar level (*P* < 0.001, Student *t* test; Figs. 5D and 7H–L).

In summary, we demonstrated that cyclin E1/CDK2 is necessary and sufficient for CENP-A Ser18 phosphorylation both *in vitro* and in cultured cells. Defective CENP-A localization at the centromere due to excessive phosphorylation of CENP-A at Ser18 enhances CIN, including chromosome missegregation and micronucleus

formation and promotes anchorage-independent growth and tumor progression (Fig. 7M).

Discussion

CIN is characteristic of most human cancers, but the mechanisms leading to CIN and its role in cancer progression are poorly understood. In the current study, we identify a novel mechanism by which *FBW7* loss promotes CIN and tumorigenesis through centromere misregulation. Specifically, we provide insight into mechanisms responsible for centromere misregulation in the cancer context and its roles during tumorigenesis, by connecting loss of *FBW7* and excess cyclin E1 with aberrant CENP-A Ser18 phosphorylation and reduced localization at the centromere. In summary, we identify an important new function for aberrant cyclin E1/CDK2 activation in cancer, distinguishable from its well-established role in the G₁–S transition (36).

Given the critical role of CENP-A in maintaining genome stability, assembly of new CENP-A at centromeres is tightly regulated to ensure faithful chromosomal segregation (48). Previous research, mostly done using other cancer cell lines, showed that the timing of CENP-A assembly is controlled by both CDK1 and CDK2 activities (19), which inhibit premature assembly of new CENP-A during S, G₂, and most of mitosis. CDK1/2 and PLK1 impact the timing of new CENP-A deposition by phosphorylating M18BP1 and HJURP in HeLa and other cell lines (18, 19, 49). Another study in HeLa suggested that phosphorylation of CENP-A at Ser68 by cyclin B1/CDK1 prevents premature CENP-A loading (20), despite some debates (21, 22). Although these studies highlighted important "normal" regulation that ensures proper centromere assembly, their roles in any clearly defined cancer model or in normal primary cells are not clear. We envision that under normal condition, cyclin E1 levels are reduced by FBW7-mediated proteolysis and remain low from G₂ to early G₁ phases, to ensure that CENP-A is not hyperphosphorylated at Ser18 (among other functions) and to permit normal CENP-A assembly during late mitosis/G₁. Here, we identify CENP-A Ser18 hyperphosphorylation after FBW7 loss or cyclin E1 overexpression in cancers as another mechanism for regulating CENP-A centromeric localization, which contributes to mitotic errors and CIN.

FBW7 loss also leads to decreased centromeric CENP-B and HEC1 levels, which could be ameliorated by cyclin E1 depletion. CENP-A Ser18 phosphorylation may also inhibit recruitment or function of other kinetochore components. For example, it is possible that pSer18 reduces CENP-A interactions with CENP-B (46, 50), despite that we did not observe significant difference in CENP-B binding between CENP-A WT versus S18D proteins or peptides (Fig. 5 and not shown). However, there is also a possibility that cyclin E1 directly regulates CENP-B and HEC1 independently from CENP-A phosphorylation, as CENP-C localization appears largely normal in FBW7^{-/-} cells. Although these important questions merit further investigations for fully understanding how FBW7 mutation or cyclin E1 overexpression impacts CENP-A assembly, we emphasize that we identified cyclin E1/CDK2 as a bona fide CENP-A Ser18 kinase, and its novel function in regulating CIN in cancer progression through centromere dysfunction relevant to many cancers with FBW7 mutation and cyclin E1 overexpression.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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FBW7 Loss Promotes Chromosomal Instability and Tumorigenesis via Cyclin E1/CDK2 –Mediated Phosphorylation of CENP-A

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