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# EWSR1 (Ewing Sarcoma Breakpoint Region 1) maintains centromere identity

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## SUMMARY

The centromere is essential for ensuring high-fidelity transmission of chromosomes. CENP-A, the centromeric histone H3 variant, is thought to be the epigenetic mark of centromere identity. CENP-A deposition at the centromere is crucial for proper centromere function and inheritance. Despite its importance, the precise mechanism responsible for maintenance of centromere position remains obscure. Here, we report a novel mechanism to maintain centromere identity. We demonstrate that CENP-A interacts with EWSR1 (Ewing Sarcoma Breakpoint Region 1) and EWSR1-FLI1 (the oncogenic fusion protein in Ewing sarcoma). EWSR1 is required for maintaining CENP-A at the centromere in interphase cells. EWSR1 and EWSR1-FLI1 bind to CENP-A through the SYGQ2 region within the prion-like domain, important for phase separation. EWSR1 binds to R-loops through its RNA-recognition motif *in vitro*. Both the domain and motif are required for maintaining CENP-A at the centromere. Therefore, we conclude that EWSR1 guards CENP-A in centromeric chromatins by binding to centromeric RNA.

### Keywords

centromere; kinetochore; CENP-A; EWSR1 (Ewing Sarcoma Breakpoint Region 1); CENP-A maintenance; EWSR1-FLI1 (Ewing sarcoma oncogenic fusion protein); centromere identity; Ewing sarcoma; phase separation

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**Author contributions:** RK and KK conceived the research, designed experiments, analyzed results, and wrote the manuscript. RK performed the major experiments. YN performed the CENP-A SNAP assays. AB purified $His_6$-EWSR1-FLI1 protein from *E. coli*. PH supervised AB, advised on the experiments, and provided Ewing sarcoma-related reagents.

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## INTRODUCTION

In humans, errant chromosome segregation may result in profound defects, including cancer, birth defects, and developmental disorders such as Down syndrome[1,2]. The centromere, a single locus on each chromosome in humans, is essential for ensuring high-fidelity chromosome transmission[3].

In most eukaryotes, the centromere harbors large arrays of repetitive DNA; however, heritability of the centromere is thought to primarily involve epigenetic modifications. In particular, CENP-A, the centromeric histone H3 variant, is considered a critical epigenetic mark[4–14]. After DNA replication, centromeric nucleosomes, including existing CENP-A, are distributed equally to the sister chromatids, and newly synthesized CENP-A is deposited at the centromere at the early G1 phase of the cell cycle in humans [15,16]. This regulation is crucial for proper centromere function and inheritance[17].

The centromeric assembly of new CENP-A depends on the Holliday junction recognition protein (HJURP), which acts as a CENP-A-specific chaperone[15,18–21]. We demonstrated that CENP-A deposition at the centromere requires ubiquitylation of lysine 124 (K124), which is mediated by CUL4A-RBX1-COPS8 E3 ligase[11,12,14]. The recruitment of HJURP to the centromere relies on the activity of the Mis18 complex (Mis18 alpha/beta and M18BP1/KNL2)[22,23], which influences the status of histone modifications and DNA methylation of centromeres[24,25]. RbAp46 and RbAp48 (homologs of Mis16) and the Mis18 complex prime the centromere for CENP-A localization[24,26].

Timely and accurate CENP-A deposition requires integrated signals from Plk1 (polo-like kinase 1) and CDK (cyclin-dependent kinase). Plk1 promotes the centromeric localization of the Mis18 complex, and CDK inhibits the assembly of Mis18 complex[27]. The RSF (remodeling and spacing factor) complex supports the assembly of CENP-A chromatin[28–30], whereas the CENP-A licensing factor KNL2 and the small GTPase-activating protein MgcRacGAP promote the stability of newly loaded CENP-A at the centromere[31,32]. More recently, we have shown that CENP-A deposition at the centromere requires the mono- or di- ubiquitylation that is mediated by the CUL4A-RBX1-COPS8 E3 ligase[11–14].

Centromeric non-coding RNA (centromeric RNA) is important for centromere integrity and functions as a kinetochore component[33–43]. The histone chaperone Facilitates Chromatin Transcription (FACT) plays an important role in CENP-A deposition at the centromere[44–47]. FACT also reassembles nucleosomes behind RNAPII together with the transcription elongation factor Spt6 [48]. Spt6 prevents the loss of old CENP-A nucleosomes[49].

The molecular mechanism of CENP-A loading to the centromere has been extensively characterized. But despite the importance of CENP-A maintenance at the centromere during interphase, the underlying mechanism of CENP-A maintenance remains obscure. Here, we report that EWSR1 (Ewing sarcoma breakpoint region 1) interacts with CENP-A and is required for CENP-A maintenance by binding to centromeric RNA in interphase.

# RESULTS

## CENP-A associates with EWSR1

While studying the potential role of CENP-A in chromosome instability of Ewing sarcoma cells, we found that CENP-A associates with EWSR1 (Ewing sarcoma breakpoint region 1). Flag-tagged CENP-A, non-ubiquitylated CENP-A mutant (CENP-$A^{K124R}$) and ubiquitin-fused CENP-A K124R mutant (CENP-$A^{K124R}Ub^{K48R}$)[11,12,14] were co-immunoprecipitated with endogenous EWSR1 in HeLa cells (Figure 1A), suggesting that CENP-A associates with EWSR1 independently of CENP-A ubiquitylation. Furthermore, we found that endogenous EWSR1 was co-immunoprecipitated with endogenous CENP-A in HT1080 and HeLa cells (Figure 1B). In addition, Flag-tagged EWSR1-FLI1 (the oncogenic fusion protein in Ewing sarcoma) (Figure 1D)[50] was co-immunoprecipitated with CENP-A in HeLa cells (Figure S1A). In addition, purified $His_6$-tagged EWSR1-FLI1 was co-immunoprecipitated with purified $His_6$-tagged CENP-A *in vitro* (Figure 1C). These data suggest that the N-terminal half of EWSR1 (Figure 1D) binds to CENP-A.

Using truncated EWSR1 proteins expressed by the TNT T7 Coupled Reticulocyte Lysate System, we determined the CENP-A binding domain of EWSR1. The SYGQ2 region, which is one of two [G/S]Y[G/S] motifs followed by a glutamine within the prion-like domain (PrLD) of EWSR1[51], was required for CENP-A interaction (Figure 1D). We also expressed truncated and mutant EWSR1-FLI1 proteins expressed in HeLa cells, and we performed immunoprecipitation with CENP-A (Figure S1B). These results support the conclusion that the SYGQ2 region is required for interaction with CENP-A.

To confirm the specificity of direct binding of EWSR1 to CENP-A *in vitro*, we used conditions in which purified CENP-A was solubilized with H4 (Figures S1C and S1D). Under these conditions, EWSR1 but not EWSR1ΔCABD (CENP-A-binding domain deletion, Δ176–245), interacted with the CENP-A-H4 complex (Figures S1C and S1D).

We also expressed truncated CENP-A proteins in HeLa cells, and then performed immunoprecipitation with EWSR1 (Figure S1E). CENP-A lacking the N-terminal 1–46 (CENP-A Δ1–46) substantially lost the ability to bind to EWSR1 (Figure S1E), suggesting that the N-terminal 46 amino acid is important for EWSR1 binding. Furthermore, CENP-A lacking 55–74 amino acids (CENP-A Δ55–74) and CENP-A lacking 75–116 amino acids lost some ability to bind to EWSR1 (Figure S1E) (see Limitations of the Study in the Discussion).

## EWSR1 is required for CENP-A maintenance at the centromere

Because EWSR1 associates with CENP-A, we examined whether EWSR1 affects CENP-A centromere localization. 48 hr after transfection, depletion of EWSR1 by siRNA abolished CENP-A signals at the centromere in interphase cells but not mitotic cells (Figures 2A–2C). Expression of a Flag-tagged siRNA-resistant EWSR1 mutant (Flag-$EWSR1^{s4886res}$) restored CENP-A signals at the centromere in EWSR1-depleted cells (Figures 2A–2C), ruling out the possibility of off-target effects associated with siRNA. Between 72 and 96 hr after transfection, presumably after completion of a few cell cycles, CENP-A signals at the centromere in mitosis also were reduced (Figures S3A and S3B).

We examined the effects of YFP-fused CENP-A expressed in the absence of endogenous CENP-A in hTERT-RPE1 cells (hTERT-RPE1 CENP-A−/− cells)[52]. Likewise, depletion of EWSR1 by siRNA abolished YFP-CENP-A signals at the centromere in interphase cells but not mitotic cells (Figures S2A, S2B and S2C).

We further tested whether other CENP proteins are affected by EWSR1 depletion. Like CENP-A, CENP-C and CENP-I (but not CENP-T) were abolished in EWSR1-depleted cells (Figure S2D), suggesting that EWSR1 is required for CENP-C-based but not CENP-T-based centromere assembly[53]. Consistent with this result, EWSR1 was immunoprecipitated with CENP-A and CENP-C but not CENP-T or CENP-I (Figure S2E).

Next, we monitored the effects of EWSR1 depletion on loading of newly synthesized CENP-A (new CENP-A) in M/G1 phase and maintenance of pre-existing CENP-A (old CENP-A) using a SNAP-tag pulse chase system in EWSR1-depleted cells (Figures 3A–3F)[15,19,32,54]. The SNAP protein tag can catalyze formation of a covalent bond to a benzyl-guanine moiety coupled to different fluorescent or non-fluorescent membrane-permeable reagents[55]. Newly incorporated pulse-labeled CENP-A-SNAP signals (new CENP-A) and preassembled pulse-labeled CENP-A-SNAP signals (old CENP-A) were reduced over time in EWSR1-depleted cells compared to Luc siRNA-treated cells (Figure 3A–3D), indicating that EWSR1 is required for CENP-A assembly and maintenance at the centromere (see Limitations of the Study in the Discussion).

To test the concept that EWSR1 is required for CENP-A maintenance in centromeric nucleosomes in interphase but not in mitotic cells, we generated EWSR1 conditional knockdown cells using a mini auxin-inducible degron system (mAID)[56], which allows prompt removal of EWSR1 from cells when auxin is added. We generated HCT116 cells (a colon tumor cell line with a relatively normal karyotype) expressing mAID-EWSR1, resulting in HCT116 [Pcmv-OsTIR1(F74GF), hygro-mAID-EWSR1/BSDN-mAID-EWSR1] (hereafter, called “HCT116 [Pcmv-OsTIR, mAID-EWSR1] cells”)[56]. Endogenous EWSR1 was not expressed because both alleles of EWSR1 were replaced with mAID-EWSR1. OsTIR1[F74G] was integrated at the safe harbor AAVS1 locus[57]. By addition of an auxin homolog (5-Phenyl-1H-Indole-3-acetic acid (5-Ph-IAA), mAID-EWSR1 was degraded within 1 h (Figure S4A). 6 h after addition of 5-Ph-IAA, CENP-A was abolished in interphase but not mitotic cells (Figure S4B). HCT116 [Pcmv-OsTIR, mAID-EWSR1] cells were arrested in mitosis with TN-16 (a microtubule inhibitor), and then 5-Ph-IAA was added. Although mAID-EWSR1 was degraded within 1 h, CENP-A signals remained at the centromere even 24 h after addition of the auxin homolog, supporting the idea that EWSR1 is required for CENP-A maintenance in centromeric nucleosomes in interphase but not mitotic cells (Figures S4C and S4D).

These results suggested that CENP-A-EWSR1 interaction may occur only in interphase. To test this concept, HeLa cells were released from double thymidine block. collected for FACS (Figure S3E) and was used for immunoprecipitation with anti-CENP-A antibodies. Less EWSR1 was co-precipitated with CENP-A in G2/M (Figures S3C and S3D). HeLa cells were then arrested, released from TN-16-induced mitosis arrest, and then used for immunoprecipitation and FACS (Figures S3F and S3G). The amount of EWSR1 co-

precipitated with CENP-A was abolished in mitosis and increased in G1 (Figure S3F), indicating that CENP-A-EWSR1 association is reduced in mitosis and restored in G1.

## CENP-A associates with centromeric RNA via EWSR1

Since centromeric RNA is important for centromere integrity[33–43] and EWSR1 is an RNA-binding protein (but not a transcription factor)[58], we tested whether EWSR1 binds to centromeric RNA *in vivo*. We performed RNA-chromatin immunoprecipitation (RNA-ChIP) [37] with anti-EWSR1 using HeLa cells. RNA-protein complexes were eluted from immunoprecipitates, and a significant amount of alpha-satellite DNA transcripts was detected by RT-PCR (Figures 4A and 4B), indicating that EWSR1 associates with centromeric RNA *in vivo*.

Because CENP-A associates with centromeric RNA[37,43], we tested whether EWSR1 is required for CENP-A–centromeric RNA interaction. We performed RNA-ChIP with anti-CENP-A antibodies using EWSR1-depleted cells. Centromeric RNA was detected by RT-PCR in CENP-A immunoprecipitates obtained from untreated cells, but not from EWSR1-depleted cells (Figures 4C–4E), suggesting that CENP-A associates with centromeric RNA in an EWSR1-dependent manner. We also examined whether EWSR1 associates with centromeric RNA via CENP-A. Centromeric RNA was detected in EWSR1 immunoprecipitates obtained from CENP-A-depleted cells (Figures 4F and 4G), suggesting that EWSR1 interacts with centromeric RNA in a CENP-A-independent manner. Therefore, we conclude that EWSR1 is required for interaction between CENP-A and centromeric RNA *in vivo*.

## CENP-A centromeric localization depends on R-loops

R-loops (three-stranded RNA/DNA structures) are located at the centromere[59]. Because CENP-A and EWSR1 associate physically with centromeric RNA *in vivo*, we tested whether reducing R-loops would affect CENP-A deposition at the centromere. We expressed RNaseH1 (which digests the RNA component of R-loops)[60] or senataxin (SETX; a putative RNA-DNA helicase postulated to dissociate R-loops)[61] to reduce the frequency of R-loops (Figures 5A–5C). CENP-A signals at the centromere were substantially reduced in RNaseH1- and senataxin-overexpressing cells (Figures 5A and 5B). Ectopic expression of RNaseH but not RNaseH$^{WKKD}$ (catalytic activity dead mutant) or RNaseH$^{D210N}$ (RNA/DNA-binding and catalytic activity dead mutant) impaired centromere localization of CENP-A (Figures S5A–S5D), confirming the specificity of the suppression. These results suggest that CENP-A is localized at the centromere in an R-loop-dependent manner.

Consistent with an earlier report[37], RNA polymerase inhibition by alpha-amanitin treatment reduced CENP-A levels at the centromere (Figures S5E–S5G). Our results support the concept that CENP-A centromere localization depends on R-loops.

To remove R-loops at the centromere specifically, we constructed Flag-RNaseH-dCas9 or Flag-RNaseH$^{D210N}$-dCas9 and guide-RNA targeting authentic centromere sequences (gRNA-CEN) or telomere sequences (gRNA-TELO). In 293T cells expressing Flag-RNaseH-dCas9, but not Flag-RNaseH$^{D210N}$-dCas9, with gRNA-CEN but not gRNA-TELO, CENP-A signals at the centromere were reduced substantially (Figures S5H–S5J), further

supporting the concept that CENP-A centromere localization depends on R-loops at the centromere.

Given these results and knowing that CENP-A interacts with centromeric RNA via EWSR1 *in vivo*, we then tested whether EWSR1 binds R-loops *in vitro*. We generated synthetic R-loop structures prepared by hybridizing an RNA strand to a DNA bubble scaffold[62]. After performing R-loop binding assays by electrophoretic mobility shift assays (EMSA) using bacterially expressed and purified His6-tagged proteins, EWSR1 bound to the R-loop structure with a significantly higher affinity than the RNA-DNA hybrid, dsRNA, dsDNA, or ssRNA (Figure 5D). This result is consistent with the previous finding that EWSR1 associates with R-loops *in vitro*[63].

Addition of EWSR1 (**lane 3** from the left in Figure 5F) but not CENP-A (**lane 2** in Figure 5F) retarded mobility of the R-loops, suggesting that EWSR1 but not CENP-A binds to R-loops. In addition, super-shifting by an anti-CENP-A antibody confirmed the formation of the CENP-A-EWSR1-R-loop complex (**lanes 4** and **5** in Figure 5F).

To confirm the specificity of binding of EWSR1 to CENP-A *in vitro*, we co-purified CENP-A with H4 (Figure S5L). An H4-CENP-A-EWSR1-R-loop complex formed (Figure S5M).

These results suggest that CENP-A associates with R-loops by binding to EWSR1 *in vitro*, consistent with the *in vivo* results.

## EWSR1-CENP-A- and EWSR1-RNA-binding domain deletion mutants

Using *in vitro* R-loop assays, bacterially expressed and purified $His_6$-EWSR and $His_6$-EWSR1ΔCABD bound to R-loops (**lanes 2** and **3** in Figure 5E, **lanes 3** and **6** in Figure 5F), but the resultant $His_6$-EWSR1ΔCABD-R-loops complex did not bind to CENP-A (**lane 8** in Figure 5F), indicating that EWSR1ΔCABD is deficient in CENP-A binding *in vitro*. $His_6$-EWSR1ΔRRM did not bind to R-loops *in vitro* (**lane 3** in Figure 5E), suggesting that EWSR1 binds to R-loops via its RNA-recognition motif (RRM). These results are consistent with the results *in vivo*.

Expression of a Flag-tagged siRNA-resistant EWSR1 mutant (Flag-$EWSR1^{s4886res}$, simplified as Flag-EWSR1*) – but not a Flag-tagged siRNA-resistant and CENP-A-binding domain (CABD) deletion EWSR1 mutant (Flag-EWSR1*ΔCABD) or a Flag-tagged siRNA-resistant (the siRNA target sequence is within the RRM) and RRM deletion EWSR1 mutant (Flag-EWSR1ΔRRM) – restored CENP-A signals at the centromere in EWSR1-depleted cells (Figures 6A, 6B and S6A). These results show that EWSR1's CENP-A binding and RNA binding are important for CENP-A deposition at the centromere.

Consistent with these results, Flag-EWSR1ΔCABD or Flag-EWSR1ΔRRM did not complement the mitotic defects induced by mAID-EWSR1 degradation (Figures S6B–S6D).

Taken together, our *in vivo* and *in vitro* data using the EWSR1 mutants support the concept that EWSR1 binds to CENP-A through the CABD and that EWSR1 binds to R-loops via the RRM (Figure 6C), both of which are important for CENP-A deposition at the centromere.

### Test of our model

As a critical test of our model, we examined whether CENP-A-RRM fusion protein (CENP-A fused with the RRM of EWSR1) would localize to the centromere in the absence of EWSR1 (Figure 7A). We generated CENP-A fused to the RRM of EWSR1 (CENP-A-RRM in Figures 7A and 7B). For functional assays, Flag-CENP-A-RRM was expressed in EWSR1-depleted cells using the pBabe retroviral vector system (Figure S7C). Flag-CENP-A-RRM, but not Flag-CENP-A, substantially restored CENP-A centromere localization when EWSR1 was depleted (Figure 7C). Further, Flag-CENP-A-RRM complemented partly the mitotic defects induced by mAID-EWSR1 degradation (Figures S6B–S6D) (see Limitations of the Study in the Discussion). These results suggest that the function of EWSR1 is to anchor CENP-A by binding to centromeric RNA, which would provide strong support for our models (Figure 6C).

To test whether the CENP-A binding domain of HJURP (the CENP-A chaperone) would functionally substitute for the SYGQ2 motifs of EWSR1, we replaced the SYGQ2 motif of EWSR1 with the CENP-A binding domain of HJURP[64] in EWSR1 to generate EWSR1*-HJURP-CABD (CENP-A-binding domain) (Figures S7A and S7B). We expressed V5-tagged-EWSR1*-HJURP-CABD in EWSR1-depleted cells using the pBabe retroviral vector system (Figure S7D). V5-EWSR1*-HJURP-CABD restored CENP-A centromere localization sufficiently when EWSR1 was depleted, while Flag-EWSR1*ΔCABD could not (Figures S7B and 7C). These results indicate that the SYGQ2 can be replaced with the CENP-A-binding domain of HJURP, providing further evidence for our model in which the role of the SYGQ2 motif is to bind CENP-A (Figure 6C).

Our model suggests that EWSR1 functions as a component of the centromere in interphase. To test this, we performed ChIP-seq experiments with anti-EWSR1 antibodies to determine where EWSR1 binds to the genome. EWSR1 bound to the centromeric regions (alpha-satellite DNA rich regions) on most chromosomes as CENP-A does (S6E) (see Limitations of the Study in the Discussion), indicating that EWSR1 is a centromere protein in interphase.

## DISCUSSION

CENP-A, the centromeric histone H3 variant, is considered the critical epigenetic mark for centromere identity[4–14]. In the current study, we discovered that EWSR1 is a novel factor required for CENP-A maintenance at the centromere in interphase. The transcription elongation factor and histone chaperone Spt6 was identified previously as a conserved CENP-A maintenance factor[49]. By contrast, EWSR1 is an RNA-binding protein but not a transcription factor[58]. Thus, EWSR1 does not appear to be involved in transcriptional activity. In fact, depletion of EWSR1 did not affect centromeric RNA levels (Figure 4C). Instead, EWSR1 associated with centromeric RNA (Figures 4A and 4F) and CENP-A *in vivo* (Figure 1). We also found that CENP-A associates physically with centromeric RNA[37] *in vivo*, depending on EWSR1 (Figure 4C). Further, our ChIP-seq analysis revealed that EWSR1 is located at the centromeric regions of chromosomes (Figure S6E). *In vitro*, CENP-A associates with R-loops via EWSR1 (Figures 5F and S5M). Thus, EWSR1 appears to function as a chaperone to maintain pre-existing CENP-A in centromeric nucleosomes by

associating R-loops (Figure 6C), which ensures long-term stability of epigenetic centromere identity.

In EWSR1-depleted cells, CENP-A remained in mitotic cells but not in interphase cells (Figure 2). One might speculate that this phenotype requires an additional loading time point, such as mitosis, in addition to M/G1. We do not exclude this possibility; however, we believe that our model in which EWSR1 maintains CENP-A at the centromere in interphase but not in mitosis could explain this phenotype. Once CENP-A is loaded at the centromere in M/G1, EWSR1 maintains CENP-A in interphase. When EWSR1 is depleted, CENP-A is maintained at the centromere when CENP-A is already in mitosis.

Phase transition or separation is a way to segregate biological macromolecules involving a change of phase or state, e.g. a transition from protein solutions to liquid-like phase-separated compartments that consist of organelles without membranes[65]. Phase transition properties of the prion-like domain in EWSR1-FLI1 (the oncogenic fusion protein for Ewing sarcoma) are critical for retargeting BAF (BRG1/BRM-associated factor) chromatin remodeling complexes[51]. The SYGQ regions are two [G/S]Y[G/S] motifs followed by a glutamine within the prion-like domain (PrLD) of EWSR1 (Figure 1D), which are required for a phase separation activity. Fusion of the SYGQ2 region to FLI1 is sufficient to recapitulate BAF complex retargeting and EWSR1-FLI1 activities[51]. Here, we found that the SYGQ2 region is required for CENP-A binding of EWSR1 or EWSR1-FLI1. Thus, phase separation caused by the SYGQ2 region of EWSR1 may contribute to centromeric chromatin remodeling for centromeric transcription (Figure 6C).

EWSR1 secures CENP-A at the centromere only during interphase but not in mitosis. Consistently, we found that EWSR1 does not interact with CENP-A in mitosis. The N-terminal end of CENP-A is important for binding to EWSR1 (Figure S1E). As there are several posttranslational modifications at the N-terminal end of CENP-A[66], they may play a role in regulation of EWSR1 interaction in mitosis. Currently we do not know why EWSR1 is not required in mitosis. We speculate that another unknown protein might have the same function in mitosis, or the mitotic chromatin structure might be sufficient to maintain CENP-A at the centromere.

The N-terminus of CENP-A was thought to be dispensable for the centromere function[67]. However, several reports support the importance of the N-terminus of CENP-A; Logsdon et al. found that a small portion of the N-terminal tail is involved in the initial recruitment of two essential constitutive centromere proteins, CENP-C and CENP-T[68]; Jing et al. found that deletion of the first 53, but not the first 29, amino terminus residues of CENP-A results in its cytoplasmic localization[69]; Sathyan et al. showed that alpha-amino trimethylation of CENP-A by NRMT is required for full recruitment of the centromere[70]. Consistent with these findings, we found that Flag-CENP-A-RRM substantially restored CENP-A centromere localization when EWSR1 was depleted (Figure 7C), suggesting that the CENP-A-EWSR1 binding, thus, the N-terminus of CENP-A, is important for CENP-A deposition at the centromere.

R-loops – nucleic acid structures consisting of an RNA-DNA hybrid and displaced single-stranded DNA – are ubiquitous in organisms from bacteria to mammals[71]. R-loops have been described at DNA replication initiation sites in bacteria. It now appears that R-loops regulate diverse cellular processes such as gene expression, immunoglobulin (Ig) class switching, and DNA repair[71]. Kabeche et al. reported that ATR associates with R-loops at the centromere in mitosis[59]. In the current study, CENP-A deposition at the centromere depended on R-loops (Figure 5. Thus, we conclude that R-loops play a fundamental role at the centromere.

Ewing sarcoma is the second most common primary malignant bone tumor in children and adolescents and is characterized by the expression of chimeric fusions of the EWSR1 (also known as EWS) and ETS family transcription factors, e.g., EWSR1-FLI1[58,72]. The response of children with metastatic or relapsed EwS is poor despite intensive multimodality therapy[73,74]. Ewing sarcomas display frequent chromosome gain or loss[75–79], which appears to be associated with lower cumulative overall survival[76]. Consistent with frequent chromosome gain or loss, we found that Ewing sarcoma cells exhibit chromosome missegregation phenotypes, i.e., chromosome instability (data not shown).

Although the exact role of chromosome instability in Ewing sarcoma is unknown, it promotes metastasis by sustaining a tumor cell-autonomous response to cytosolic DNA. Furthermore, suppression of chromosome instability substantially reduces metastasis[80]. Thus, determining the mechanism of chromosome instability in Ewing sarcoma could contribute to development of therapies to suppress metastasis. In this study, we found that EWSR1-FLI1 and EWSR1 bind to CENP-A. EWSR1-FLI1 is a transcription factor but not an RNA-binding protein because of loss of its C-terminal RNA-binding domain [58,72]. Since EWSR1-FLI1 binds to CENP-A but not centromeric RNA, it should work as a dominant negative mutant against EWSR1 for CENP-A maintenance. Further work is required to test these hypotheses.

EWSR1-FLI1 impairs the localization of Aurora B kinase to the midzone during anaphase, which induces chromosome instability[81–83]. However, how EWSR1 functions with Aurora B is not known. Our finding of EWSR1 function in CENP-A maintenance could explain this phenotype because CENP-A is phosphorylated by Aurora B kinase and plays a role in completion of cytokinesis[84].

## Limitations of the Study

Deleting the N-terminal 1–46 (CENP-A Δ1–46) substantially abolished the binding ability of CENP-A to EWSR1 (Figure S1E), indicating that the N-terminal 46 amino acid form of CENP-A is required for EWSR1 binding. However, removing the 55–74 or 75–116 amino acids of CENP-A reduced its binding ability to EWSR1 (Figure S1E). Since CENP-A is a small protein (140 amino acids), deletion of dozens of amino acids might change its structural conformation and affect its binding ability. Currently, we are determining the structure of CENP-A-EWSR1 in the presence of nucleosomes by cryo-electron microscopy, which would determine the EWSR1-binding domain of CENP-A.

Results of our CENP-A-SNAP assays suggest that EWSR1 is required for both CENP-A assembly in M/G1 and maintenance in interphase at the centromere (Figure 3). However, if EWSR1 is required for maintaining CENP-A at the centromere, depletion of EWSR1 would delocalize assembled CENP-A from the centromere. Therefore, we surmised that the primary function of EWSR1 might be to maintain CENP-A at the centromere in interphase. Our conditional degradation experiments using mAID-EWSR1 showed that EWSR1 is required for maintaining CENP-A at the centromere in interphase but not in mitosis (Figure S4), which supports our hypothesis.

Flag-CENP-A-RRM restored about 80% of CENP-A centromere localization when EWSR1 was depleted (Figure 7C), supporting our model of EWSR1 function at the centromere. However, Flag-CENP-A-RRM complemented about 60% of the mitotic defects induced by mAID-EWSR1 degradation (Figures S6B–S6D). This difference suggests that the mitotic function of EWSR1 might not be solely to connect CENP-A to centromeric RNA.

Our ChIP-seq experiments clearly indicate that EWSR1 binds to the centromeric regions of the genome. However, we failed to detect centromeric localization of EWSR1 by immunofluorescence microscopy although we tried several different conditions. As EWSR1 seems abundant, in future experiments, appropriate extraction might be necessary to visualize the centromeric localization in fixed cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Inclusion and diversity:

We support inclusive, diverse, and equitable conduct of research.

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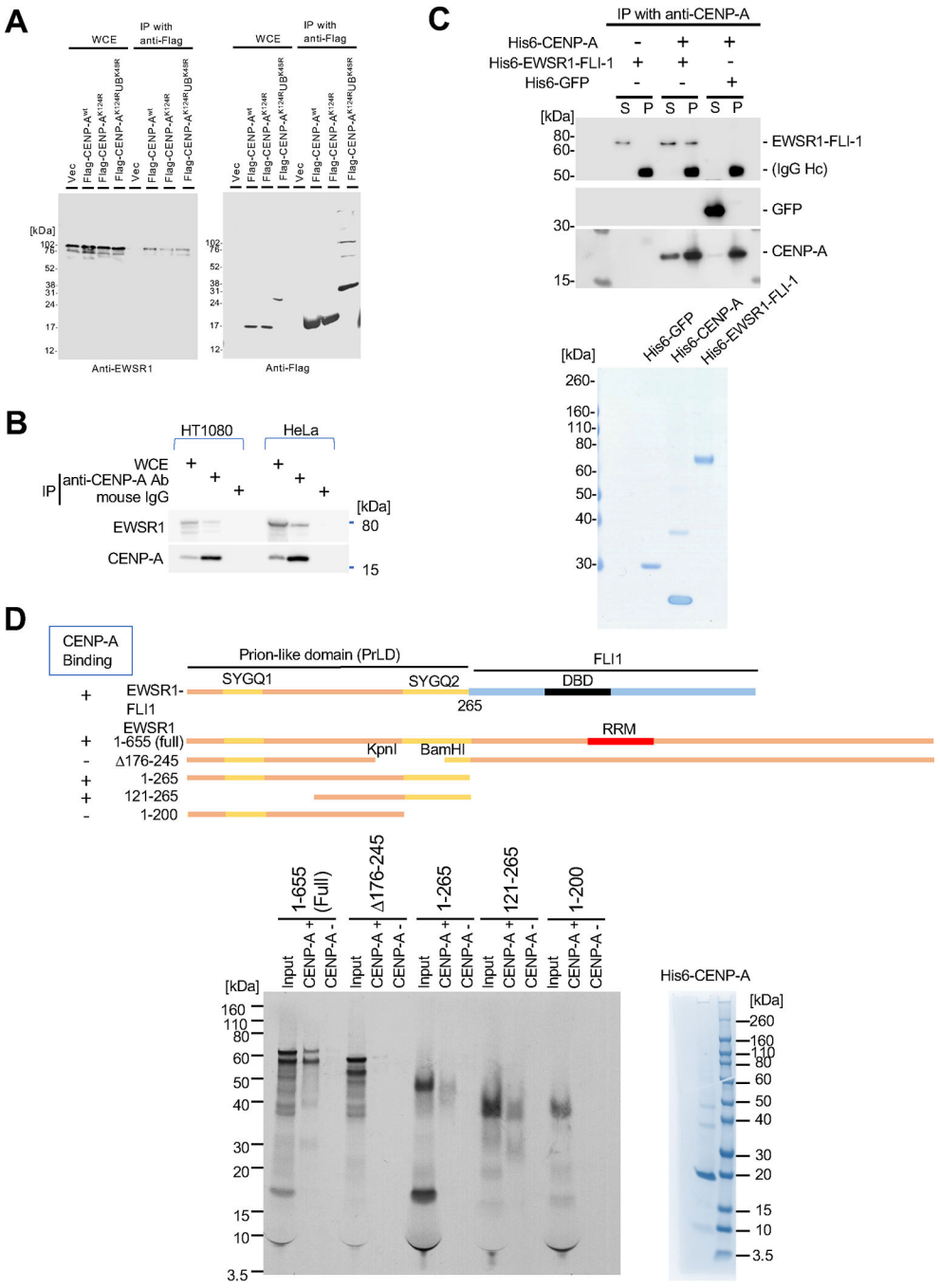


**Figure 1. CENP-A associates with EWSR1.**

(**A**) Flag-CENP-A associates with EWSR1. 293T cells were transfected with plasmid vectors expressing the indicated Flag tagged CENP-A proteins or an empty vector (Vec). Cell lysates prepared 48 h after transfection were subjected to immunoprecipitation with anti-Flag antibody. Immunoblotting was performed with anti-EWSR1 antibody (left panel) and anti-Flag antibody (right panel). WCE: whole cell extract. EWSR1 was co-precipitated with Flag-CENP-A WT, Flag-CENP-A K124R (non-ubiquitylated mutant) and Flag-CENP-A K124R UbK48R (mono-ubiquitin fusion CENP-A). As ubiquitylated forms are only ~5%

of the total proteins, they are not visible in this panel. (**B**) Endogenous CENP-A associates with endogenous EWSR1. CENP-A was immunoprecipitated from WCE prepared from HT1080 and HeLa cells using CENP-A-specific monoclonal antibody or mouse IgG. Immunoprecipitates and 5% input (WCE) were separated on SDS polyacrylamide gel, then transferred to PVDF membrane. EWSR1 and CENP-A were immunoblotted with anti-EWSR1 rabbit polyclonal antibody and anti-CENP-A mouse monoclonal antibody, respectively. EWSR1 was co-immunoprecipitated with CENP-A in both HT1080 and HeLa cells. (**C**) CENP-A binds to EWSR1-FLI1 directly. $His_6$-tagged recombinant proteins were expressed in *E. coli* and purified using Ni-NTA beads. In the bottom panel, purified proteins were separated and visualized by staining with Simply Blue. To test the direct interaction of $His_6$-EWS-FLI-1 and $His_6$-CENP-A, purified proteins were incubated in RIPA buffer [0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 50 mM Tris-HCl(pH8.0), 150 mM NaCl] for 1 hr. Then the protein complex was immunoprecipitated with anti-CENP-A antibody bound to protein A sepharose. Immunoprecipitates (P) and supernatants (S) were separated, then subjected to immunoblotting. $His_6$-EWS-FLI-1 was detected in the immunoprecipitates in a CENP-A-dependent manner. $His_6$-GFP, which was used as a negative control, was not detectable in CENP-A immunoprecipitates. (**D**) Delimitation of the CENP-A association domain on EWSR1. Top: schematic representation of full-length and truncated proteins used in pull down experiments. All proteins were expressed in rabbit reticulocytes (*in vitro* transcription coupled translation system) in the presence of $S^{35}$-methionine as substrate. Bottom: CENP-A pull down assay. Reticulocyte lysate containing $S^{35}$-labeled proteins were mixed with bacterially expressed and purified $His_6$-CENP-A protein (right panel) bound to Ni-NTA beads (CENP-A+). The same amount of reticulocyte lysate was mixed with only Ni-NTA beads as negative control (CENP-A−). After incubation at room temperature for 1h, the bead-bound fraction was washed and eluted. Eluted proteins were separated on SDS-polyacrylamide gels. The gels were dried and subjected to autoradiography to detect the $S^{35}$-labeled proteins.

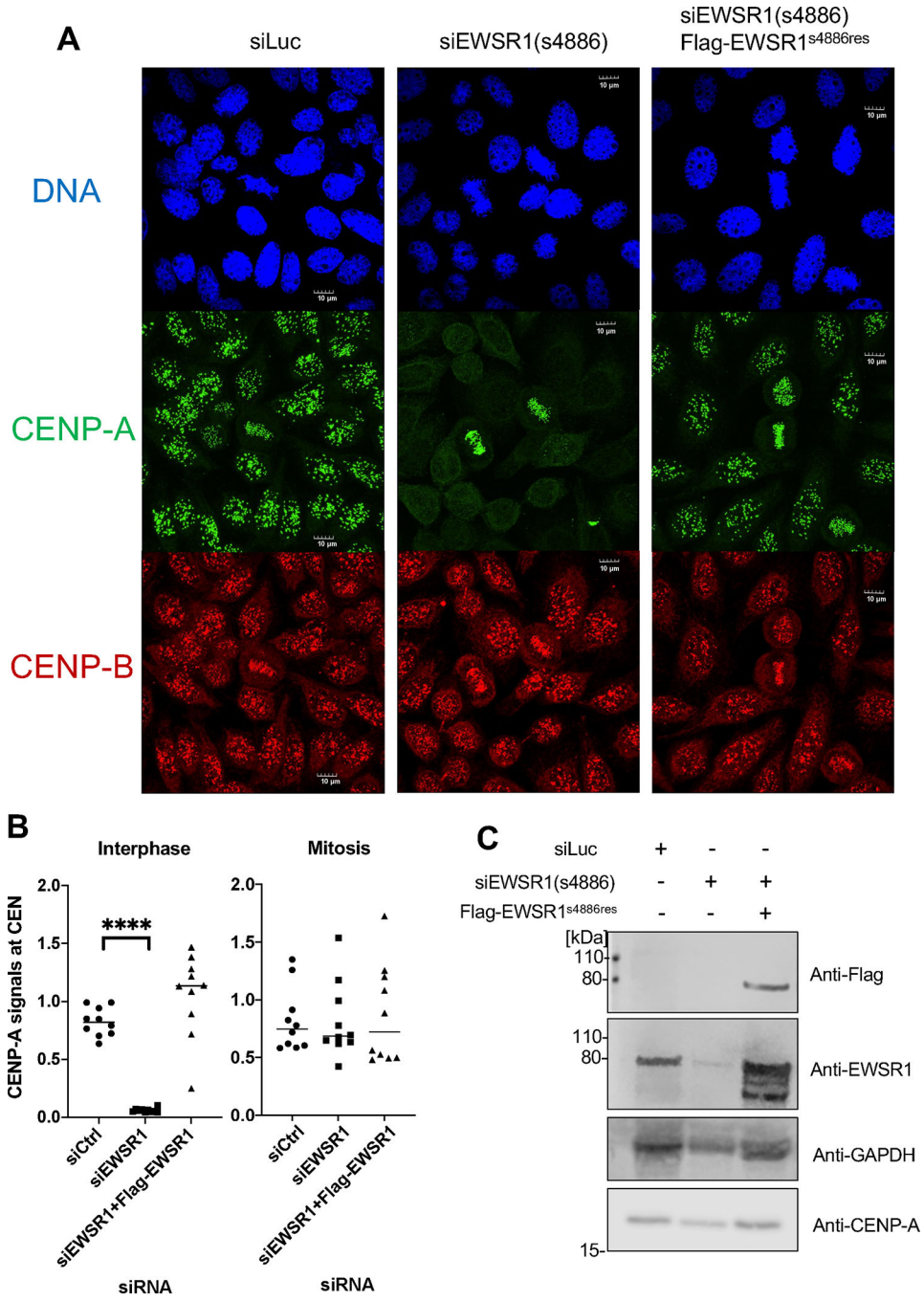


**Figure 2. Depletion of EWS reduces CENP-A centromere signals.**

HeLa cells grown on cover glass were transfected with control RNA (siLuc), siRNA targeting EWSR1 (siEWSR1) or siRNA targeting EWSR1 (siEWSR1) + plasmid vector expressing Flag-tagged siRNA resistant EWSR1 (EWSR1$^{s4886res}$). As CENP-B binds to alpha-satellite DNA and as the centromere localization of CENP-A is not dependent on CENP-B, CENP-B signals can serve as a centromere position control. (**A**) 48 hr after transfection, cells were fixed with PFA (or acetone) and immunostained with anti-CENP-B antibody and anti-CENP-A antibody. Nuclei were visualized by staining with DAPI. Images

were captured using an Olympus FV3000 confocal laser scanning microscope equipped with an UPLSAPO 100x oil immersion lens and operated with FluoView FV1000 Imaging Software. CENP-A localization at the centromere (marked with CENP-B) in interphase was abrogated by depletion of EWSR1. (**B**) Signal intensity of CENP-A at centromeres marked with CENP-B in cells depleted of EWSR1 (siEWSR1) was measured using Volocity 6.3 image analysis software (Perkin Elmer), and the ratio to signal in control cells (siLuc) was calculated. ****$p < 0.00001$ compared with Luc siRNA-treated cells (Student's t test) (**C**) HeLa cells transfected with indicated siRNAs and plasmid were collected 48 hr after transfection and were subjected to Western blotting with anti-Flag antibody, anti-EWSR1 antibody, anti-CENP-A antibody, or anti-GAPDH antibody. The signal intensity of each band was analyzed using the Odyssey CLx infrared imaging system (Li-Cor Biosciences).

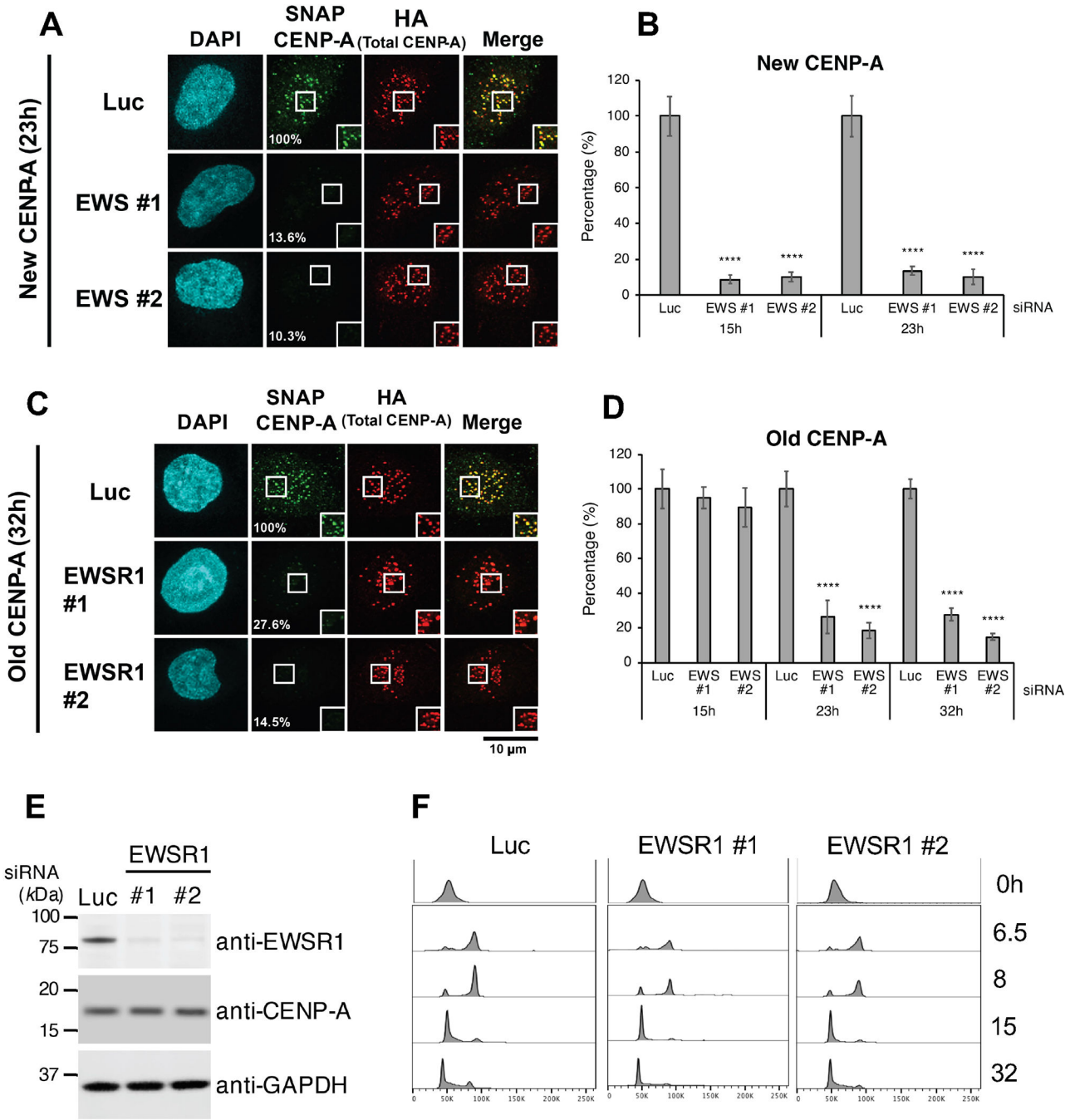


**Figure 3. EWSR1 is required for CENP-A maintenance at the centromere.**

A significant amount of newly incorporated CENP-A (**A**) and preassembled CENP-A (**C**) are lost in the absence of EWSR1. The average intensity of signal (percent) at centromeres in the cell, normalized with Luc siRNA-treated cells at each time point, is listed in the lower left corner of panels showing SNAP-CENP-A-stained cells. Representative images for 32h time point after release from thymidine are shown. Scale bar, 10 μm. (**B**), (**D**) Quantification of SNAP-tag-labeled CENP-A signals from the experiment shown in the left panel. Signals of interphase cells were quantified per experiment and the mean percentages

(±SEM) are shown. ****$p < 0.00001$ compared with Luc siRNA-treated cells at each time point (Student's t test). (**E**) Western blot analyses of samples subjected to SNAP assays in Figures 3A and **B** to confirm siRNA-mediated knockdown of EWSR1. (**F**) FACS analysis of cell cycle progression of HeLa cells released from thymidine block. HeLa cells were transfected with siRNA targeting luciferase (Luc) or EWSR1 (EWSR1#1/#2) prior to thymidine block. Cells at 15 h, 23 h, and 32 h after release from thymidine block were subjected to the experiments shown in Figures 3A and C.

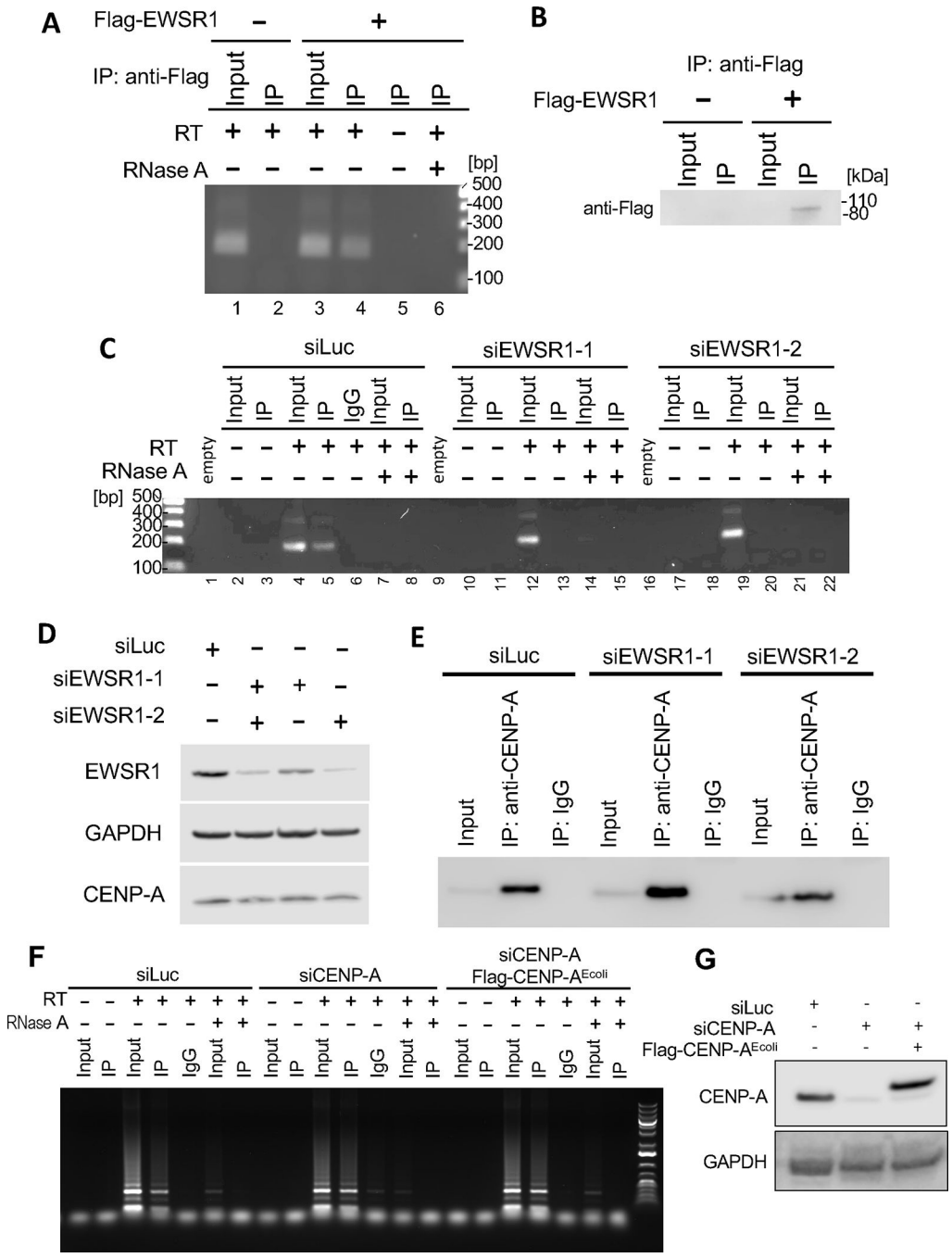


**Figure 4. CENP-A associates with centromeric RNA via EWSR1.**

(**A**) RT-PCR detection of centromere RNA associated with EWSR1 following EWSR1-mediated RNA-ChIP. HeLa cells were transfected with Flag-EWSR1 expression vector. 48 hr after transfection, Flag-EWSR1 was immunoprecipitated with anti-Flag antibody (M2) (Flag-EWSR1+, IP). RNA was extracted from the input fraction and immunoprecipitation fractions by proteinase K treatment followed by Trizol/chloroform extraction, then subjected to RT-PCR for detection of centromere RNA using alphoid DNA-specific primers. Untransfected HeLa cells were treated in the same manner and the immunoprecipitates

(Flag-EWSR1 −, IP) were used as negative controls. A comparable amount of centromere RNA was detected as 171-bp PCR product from input fractions with or without Flag-EWSR1. Centromere RNA was specifically detected in Flag-EWSR1 immunoprecipitates (IP). The 171-bp PCR product was not detectable when RNA samples were treated with RNase A prior to RT-PCR reaction (RNase A +), or when the RT-PCR reaction was performed without reverse transcriptase (RT −), suggesting that the PCR product was derived from co-immunoprecipitated RNA but not DNA. (**B**) Validation of Flag-EWSR1 immunoprecipitation with anti-Flag antibody. (**C**) EWSR1 knockdown by siRNA abrogates interaction between CENP-A and centromeric RNA. Detection of CENP-A− associated centromeric RNA by RT-PCR following CENP-A-mediated RNA-ChIP. HeLa cells were treated with siRNAs targeting EWSR1 (siEWSR_1 and siEWSR_2) or luciferase (siLuc) for control. 48 hr after transfection, cells were collected and subjected to immunoprecipitation with anti-CENP-A mouse antibody bound to Dynabeads-goat anti-mouse IgG. Input and Immunoprecipitated fractions (IP) were treated with proteinase K and RNA was extracted with TRIzol:chloroform, and treated with DNase turbo. Primers specifically amplifying a 171 bp of alphoid DNA monomer were used for One-Step RT-PCR (RT +), RT −: reverse transcriptase was not added to the reaction mixture. RNase A +: Extracted RNA was incubated with 0.2 $\mu$g/$\mu$L of RNase A at 37C for 15 min before DNase treatment. IgG: immunoprecipitation with mouse IgG used as a negative control for IP. (**D**) Validation of EWSR1 knockdown by siRNAs. A portion of cells treated with indicated siRNAs were subjected to Western blotting with anti-EWSR1, anti-GAPDH, and anti-CENP-A antibodies. EWSR1 protein was diminished by both siEWSR1_1 and siEWSR1_2. (**E**) Validation of immunoprecipitation with anti-CENP-A antibody. A small portion of the input (Input), immunoprecipitates with anti-CENP-A antibody (IP: anti-CENP-A) and with mouse IgG (IP: IgG) used for RNA-ChIP were immunoblotted with anti-CENP-A antibody. The amounts of CENP-A protein immunoprecipitated from HeLa cells treated with siEWSR1_1 and siEWSR1_2 were comparable to those treated with siLuc. (**F**) RT-PCR mediated detection of alphoid-RNA co-immunoprecipitated with EWSR1 in HeLa cells transfected with siRNA targeting luciferase (siLuc) or CENP-A (siCENP-A). Neither depletion of CENP-A by siRNA or add-back of Flag-CENP-A affected the amount of alphoid-RNA co-immunoprecipitated with EWSR1. (**G**) Western blot analyses of the samples subjected to RNA-immunoprecipitation and RT-PCR shown in **F** to confirm siRNA-mediated knockdown of endogenous CENP-A and expression of Flag-CENP-A$^{Ecoli}$. CENP-A$^{Ecoli}$ has multiple silence mutations, including those within the siRNA targeting sequence, and therefore is resistant to siRNA.

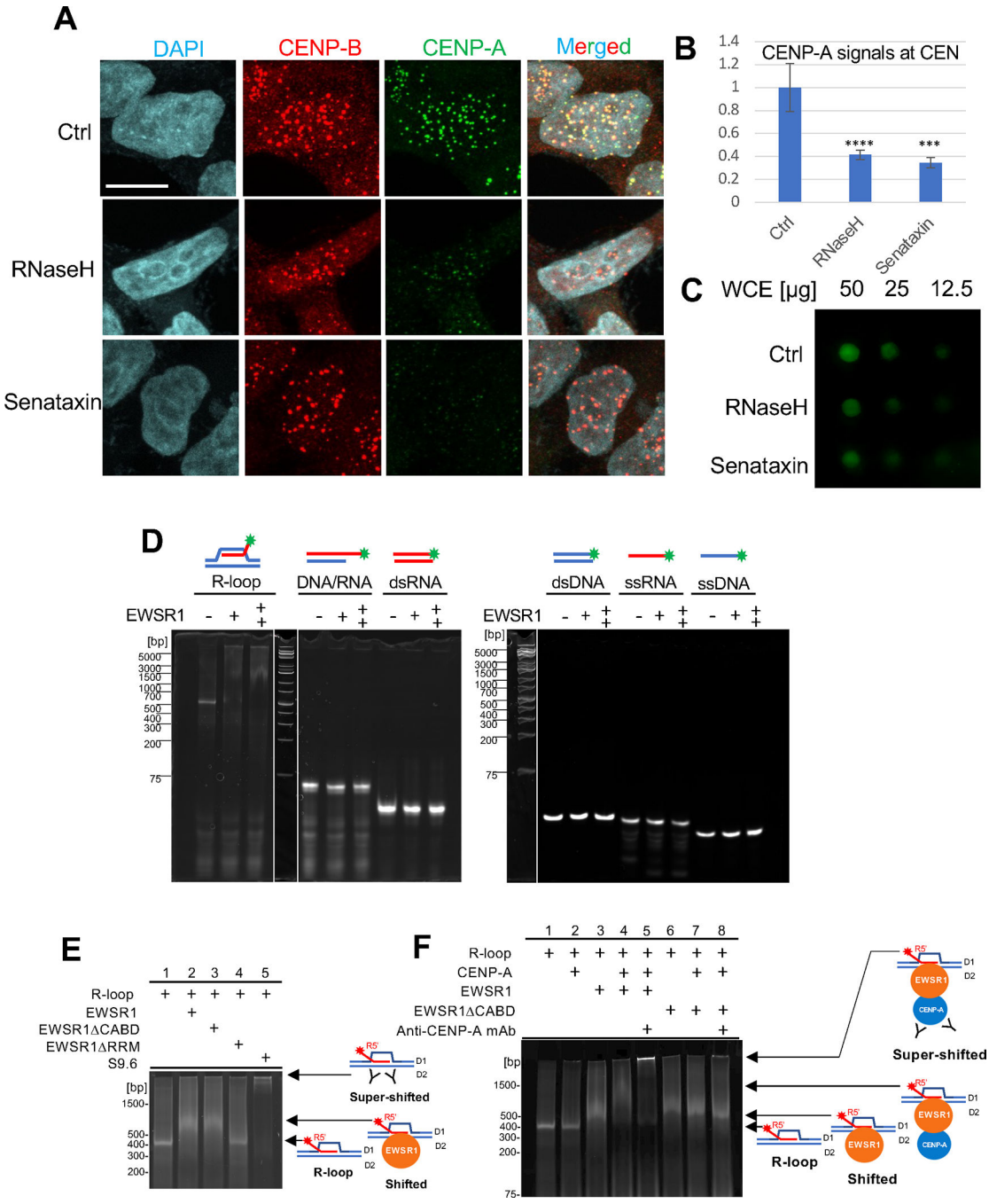


**Figure 5. Ectopic expression of RNaseH or senataxin impairs centromere localization of CENP-A**

(**A**) HeLa cells were transfected with GFP-RNaseH or senataxin-Halo expression vectors and fixed with 4% formaldehyde 48 hr after transfection. CENP-A and CENP-B were immunolabeled with Alexa Fluor Plus 647 and Alexa Fluor Plus 555, respectively. DNA was counterstained with DAPI. Images were captured with an FV3000 confocal microscope (Olympus); representative single cell images are shown. Scale bar: 10μm. (**B**) CENP-A signals at the centromere (marked with CENP-B) were measured by Fiji image analysis software, normalized by CENP-B signals. Relative intensity of centromeric CENP-A

signal in cells expressing GFP-RNaseH (RNaseH) or senataxin-Halo (senataxin) to that in untransfected cells (Ctrl) is shown. ***p < 0.001 and ****p < 0.00001 (Student's t test) (**C**) Validation of R-loop reduction by expression of RNaseH or senataxin. Indicated amounts of whole cell lysates (WCE) prepared from transfected cells were spotted on an Immobilon-FL PVDF membrane, and immunoblotted with S9.6, an anti-R-loop antibody. Expression of both RNaseH and senataxin reduced S9.6 signals ~60%, suggesting that R-loops were reduced. (**D**) *In vitro* R-loop binding assay. A model R-loop molecule, as well as other control nucleotide molecules (DNA/RNA hybrid, dsRNA and dsDNA) were generated as described previously. Briefly, synthetic oligonucleotides labeled with fluorescein at 5' end and its complementary strand were mixed and annealed. For gel mobility shift assays, 6 pmoles of nucleotide molecule was mixed with 7 pmoles (+) or 14 pmoles (++) of EWSR1 and incubated. Nucleoprotein complexes were separated on native-polyacrylamide gels and fluorescent signals detected by using Chemi Doc MP (BioRad). (**E**) RNA recognition motif but not CENP-A binding domain of EWSR1 is required for interaction with R-loop. Gel mobility shift assays revealed that the EWSR1 and CENP-A-binding domain deletion mutant (ΔCABD), but not the RNA recognition motif deletion (ΔRRM), binds to the model R-loop substrate. S9.6 was used as a positive control. (**F**) *In vitro* R-loop binding assay. A 3-stranded model R-loop substrate (R-loop) was assembled using two 90 mer-DNA oligonucleotides (D1 and D2) and a 50-mer RNA oligonucleotide conjugated with fluorescein at the 5' end, R5. $His_6$-CENP-A, $His_6$-EWSR1, and $His_6$-EWSR1ΔCABD were bacterially expressed and purified on Ni-NTA beads. R-loop substrate and indicated proteins were incubated for 30 min at room temperature. CENP-A antibody was added 15 min after the beginning of incubation.

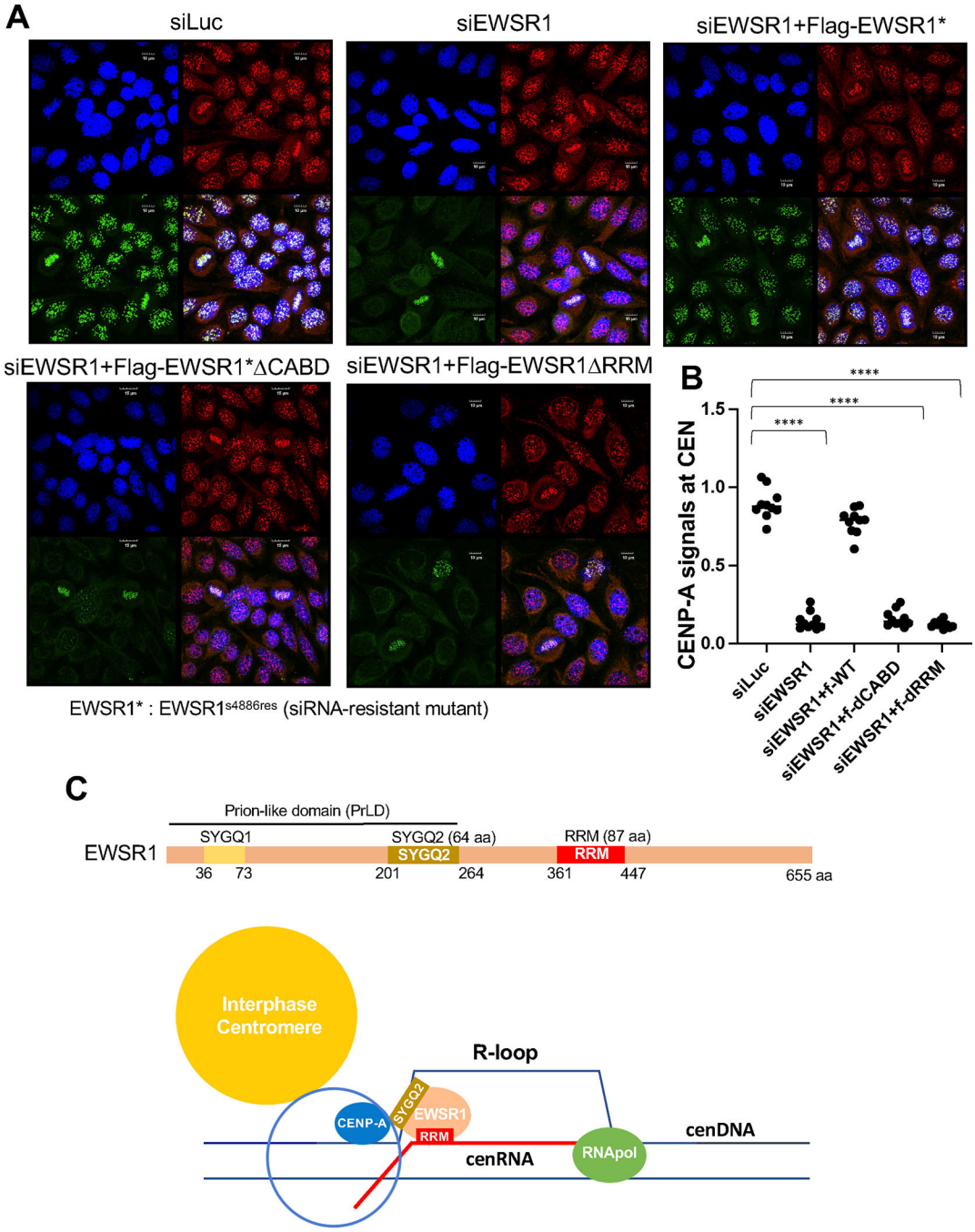


**Figure 6. CENP-A-binding domain deletion mutant of EWSR1 and RNA-recognition motif deletion mutant of EWSR1 cannot rescue mislocalization of CENP-A.**

(**A**) HeLa cells were transfected with siRNA targeting luciferase (siLuc) or EWSR1 (siEWSR1). 24 hr after transfection with siRNAs, cells were infected with retrovirus expressing indicated proteins, and incubated for an additional 24 h. Cells were collected and divided into two portions. One portion was subjected to immunoblotting to confirm the reduction of endogenous EWSR1 and expression of flag-proteins (Fig S5). Another portion was seeded on cover glass and incubated for 12 hr, then fixed with acetone, and

immunostained with anti-CENP-A antibody (green) and anti-CENP-B (red) antibodies. DNA was visualized by staining with DAPI (blue). Representative confocal microscopy images are shown. (**B**) Fluorescent signals of CENP-A and CENP-B in cells shown in (A) were measured using Fiji (NIH Image) software. Relative intensity of CENP-A at the centromere per nuclei of interphase cells was plotted. ****$p < 0.00001$ (Student's t test) (**C**) **Model of EWSR1 function at the centromere in interphase cells.** EWSR1 binds to CENP-A and R-loops and interphase centromere complexes are built on the CENP-A-containing nucleosomes (blue circle).

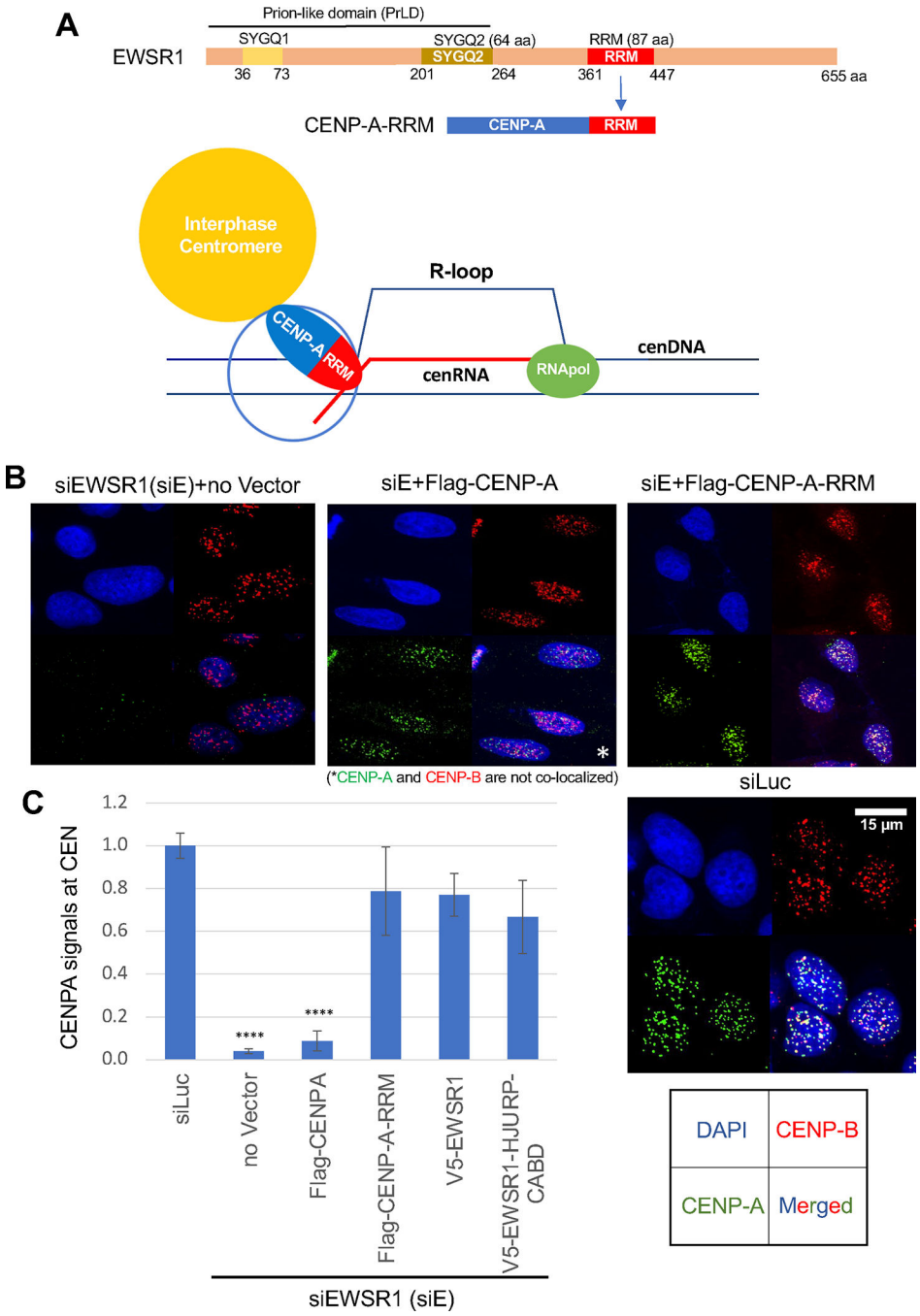


**Figure 7. Complementation assays.**

(**A**) Schematic illustration of EWSR1 and CENP-A-RRM (CENP-A fused with the RNA-recognition motif [RRM] domain of EWSR1 at the C-terminal end). CENP-A-RRM is expressed in EWSR1-depleted cells. In the absence of EWSR1, the CENP-A-RRM can bind to centromeric RNA (cenRNA) via the RRM. (**B**) **Restoring CENP-A centromere localization by expression of chimera or fusion proteins.** HeLa cells were transfected with siEWSR1(siE)+ no vector, siEWSR1+Flag-CENP-A, siEWSR1+Flag-CENP-A-RRM, siEWSR1*+V5-EWSR1 (positive control), siEWSR1+V5-EWSR1*-HJ(HJURP)-CABD,

or siLuc only and fixed 48 h after transfection. Immunofluorescence analyses and quantification of CENP-A signals at the centromere were performed. Flag-CENP-A showed dot-like green signals, but they were not colocalized with CENP-B (siE+Flag-CENP-A). Since Flag-CENP-A was expressed in the presence of endogenous CENP-A in EWSR1-depleted cells, it is likely that excess amounts of CENP-A, which can be present in nuclei but not at centromeres, may have randomly accumulated in nuclei (siE+Flag-CENP-A). Most of the Flag-CENP-A-RRM signals were colocalized with CENP-B (siE+Flag-CENP-A-RRM). (**C**) CENP-A signals at the centromere (marked with CENP-B) were measured by Fiji image analysis software and relative intensity was normalized by using CENP-B signals. ****$p < 0.00001$ (Student's t test)