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Supplemental Information

BUB1 and CENP-U, Primed by CDK1, Are the Main PLK1 Kinetochore Receptors in Mitosis

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Supplementary material for

BUB1 and CENP-U, primed by CDK1, are the main PLK1 kinetochore receptors in mitosis

by Priyanka Singh *et al.*

Including

Legends to Supplementary Figures 1-7

Supplementary Figures S1 to S7

Supplemental Figure Legends

Figure S1 *Additional localization and biochemical experiments* (associated with Figures 1-2)

(**A**-**F**) Cells cultured like in Figure 2A-C were treated with the indicated drugs and then processed at the indicated timepoints for either immunofluorescence (A-F) or western blotting analysis (G). Dot plots (A-E) show individual kinetochore quantification of the indicated protein/CREST intensity ratios normalized by control. Red lines represent median and interquartile range respectively. The following range of kinetochores number were scored for these conditions: A) 122- 441, B) 163-751, C-D) 86-240, E) 76-285. Between 5 and 15 cells per condition were analyzed. (**G**) Western blotting analysis of cells extracts prepared from cells in (A-E) and probed for the indicated proteins. (**H**) Western blots showing BUB1 expression status in mock and BUB1 RNAi HeLa cells. Tubulin was used as loading control. (**I**) Representative images of HeLa Mock and BUB1 RNAi cells stained for DAPI, PLK1 and H2A-pT120. Scale bar on each image is 5 μm. (**J**) Dot plots representing intensity ratios of PLK1 over CREST (left) and AurB-pT232 over CREST (right) for individual kinetochores under the indicated conditions. A total 382-1298 individual kinetochores belonging to 7-17 different cells were scored. The red line indicates mean ± standard deviation in each case.

Figure S2 *Additional localization and phosphorylation experiments* (associated with Figures 1-3)

(**A**) SDS-PAGE analysis of the indicated samples stained with Coomassie or Pro-Q™ Diamond. This is the same set of gels shown in Figure 1G, but providing a side-a-side view of each sample. (**B**) HeLa cells were released from double-thymidine block into nocodazole containing media (3.3 μM) to synchronize them in pro-metaphase stage of the cell cycle. Cells were exposed to either DMSO (control) or hesperadin (500 nM) for 90 min, in the presence of the proteasome inhibitor MG132 (10 μM) before fixation. Representative cells were immunostained for DAPI (DNA marker), H3pS10 (Aurora B activity marker), PLK1, and CREST (kinetochore marker). (**C**) Dot plots representing PLK1/CREST kinetochore intensity ratios for the indicated conditions. Red lines indicate median with interquartile range. A total of 220 kinetochores for DMSO-treated cells and 102 kinetochores for hesperadin-treatment, from 3-4 different cells were scored. (**D**) Schematic representation of CENP-B (CB)-INCENP-EGFP used in rescue experiments. It has the CENP-B DNA binding domain fused to INCENP protein from amino acids 48 to 918, followed by an EGFP-tag at the C-terminus. (**E-G**) HeLa cells cultured like in Figure 2E were

either mock treated or depleted for BUB1-RNAi alone or in presence of doxycycline-inducible (10ng/ml) CB-INCENP-EGFP. Cells were then prepared for either western blotting (E) or immunofluorescence analysis (F-G) and probed for the indicated antibodies. Scale bar = 5 μm. Dot plots (G) show individual kinetochore quantification of control normalized PLK1/CREST and pT232/CREST intensity ratios. Red lines represent median and interquartile range respectively. A range of kinetochores number between 275-500 and 342-438 was scored for Plk1 and AurB-pT232 respectively. (**H**) Representative images of prometaphase HeLa cells expressing doxycycline-inducible CB-INCENP-EGFP in BUB1 RNAi background. Cells were treated with DMSO or hesperadin (500 nM) for 90 minutes in the presence of MG132 (10 μM), stained for DAPI and PLK1. EGFP represent expression status of CB-INCENP-EGFP. Scale bar = 5 μm. Small insets show zoomed image of same region in PLK1 and EGFP channels. (**I**) Representative images of prometaphase HeLa cells treated for RNAi against CENP-Q and stained for CENP-Q and BUB1. (**L**) Dot plots representing BUB1/CREST kinetochore intensity ratios after CENP-Q RNAi. Error bars represent +/- standard error of mean.

Figure S3 ***Additional SEC runs*** **(associated with Figures 1 and 3)**

(**A**) Elution profile and SDS-PAGE analysis of a SEC run on a Superose6 5/150 column of the interaction of MBP-PLK1^{PBD} with the BUBR1:BUB3 complex (5 μM each) in presence of the indicated priming kinases (marked in red; used at 1/20 ratio). MBP-PLK1^{PBD} control elution profile and SDS-PAGE are also displayed in Figure 1H. (**B**) Elution profiles and SDS-PAGE analysis of SEC runs on a Superose 6 increase 5/150 column of MBP-PLK1^{PBD} alone (5 μM) (grey curve) or of mixtures of rKT (5 μM) with MBP-PLK1^{PBD} (5 μM) in presence or absence of the priming kinases PLK1, CDK1:CyclinB, Aurora B, BUB1 or their combination. (**C-D**) Elution profiles and SDS-PAGE analysis of SEC runs on a Superdex 200 increase 5/150 column of a mixture of CENP-OPQUR complex (5 μM) (B) or CENP-OP (5 μM) (C) and MBP-PLK1^{PBD} (5 μM) with or without the four mitotic kinases CDK1:Cyclin-B, PLK1, Aurora B, and BUB1 used at 20:1 ratio. Orange arrows highlight MBP-PLK1^{PBD} in each gel. The MBP-PLK1^{PDB} control elution profile and SDS-PAGE in panel C are also displayed in Figure 3C.

Figure S4 ***Role of MPS1 and its inhibition in PLK1 kinetochore recruitment*** **(associated with Figure 2)**

(**A, C, E**) Representative images of pro-metaphase synchronized HeLa cells treated with either DMSO, reversine (500 nM), okadaic acid (100 nM), or reversine plus okadaic acid for 90 min in the presence of nocodazole (3.3 μM) and proteasome inhibitor MG132 (10 μM). Cells were immunostained for CREST, DAPI, and either BUB1 (A), MPS1 (C) or PLK1 (E). Scale bar = 5 μm. (**B, D, F**) Dot plots representing control normalized intensity ratios of the indicated protein over CREST for individual kinetochores of cells from A, C and E respectively. The following range of kinetochores number were scored for these conditions: B) 122-241, D) 146-532, F) 134-410. Between 5 and 15 cells per condition were analyzed. (**G**) Representative images of pro-metaphase synchronized HeLa cells treated with either DMSO, hesperadin (500 nM), okadaic acid (100 nM), or hesperadin plus okadaic acid for 90 min in the presence of nocodazole (3.3 μM) and proteasome inhibitor, MG132 (10 μM). Cells were immunostained for Aurora B, CREST, and DAPI. Scale bar = 5 μm. (**H**) Dot plots representing control normalized intensity ratios of Aurora B over CREST for individual kinetochores. Red lines indicate median with interquartile range. Between 211 and 287 kinetochores were scored for each condition. Between 5 and 15 cells per condition were analysed. (**I**) Phosphorylation state of the input proteins of the SEC experiment in (J) was assessed by SDS-PAGE followed by Coomassie or Pro-Q™ Diamond staining. (**J**) Elution profile and SDS-PAGE analysis of analytical SEC runs on a Superdex 200 increase 5/150 column of Alexa488-labelled MBP-PLK1^{PBD} alone (orange) and of mixtures of MBP-CENP-U$^{58-114}$ with fluorescent MBP-PLK1^{PBD} (5 μM) in presence of the indicated priming kinases. Orange arrows highlight MBP-PLK1^{PBD} in each gel.

Figure S5 *Additional SEC runs* (associated with Figure 3)

(**A**) Schematic representation of the recombinant kinetochore particles containing 22 subunits (rKT, for recombinant kinetochores) used in this study, containing the majority of inner and outer kinetochore proteins. Black arrows depict direct interactions and kinetochore recruitment dependencies. (**B**) Phosphorylation status of the inputs of the SEC experiment in Figure S3B were assessed using SDS-PAGE with Pro-Q™ Diamond staining followed by Coomassie staining. (**C**) As in (**B**) for the experiments in (D). (**D**) Elution profile and SDS-PAGE analysis of SEC runs on a Superdex 200 increase 5/150 column of CENP-OPQUR (5 μM) with MBP-PLK1^{PBD} (5 μM) with or without PLK1, CDK1:CyclinB, Aurora B and BUB1 alone or in combination. In-gel fluorescence of MBP-PLK1^{PBD} in the SEC fractions analyzed by SDS-PAGE is also shown. Orange arrows highlight MBP-PLK1^{PBD} in each gel.

Figure S6 ***RNAi-based depletions of CENP-OPQUR subunits and additional ectopic localization experiments*** **(associated with Figures 3-5)**

(**A**) Western blot of total cell lysates of HeLa Flp-In TReX cells stably expressing doxycycline inducible GFP-CENP-Q in mock and CENP-Q RNAi condition. The blot represents GFP-CENP-Q and Tubulin (loading control). (**B**) Graphical representation of HeLa cells expressing PLK1 and GFP-CENP-Q kinetochore intensity signal correlation in mock vs CENP-Q RNAi condition. (**C**) Schematic representation of the components of the CENP-QU complex. Coiled-coil (CC) predictions calculated with program COILS (Lupas et al., 1991) showing each subunit with partial or complete CC content. (**D**) Representative images showing U2OS-lac-O cells in Interphase, transiently expressing Lac-I-EGFP-CENP-U full length, FL (1-418 aa), or deletions (with 1-114 or 115-418 aa). Cells were immunostained for DAPI, PLK1 and CREST. EGFP represents CENP-U localized at ectopic location on chromatin. White dotted circle highlights ectopic location of CENP-U and respective PLK1 signal. (**E**) Representative images showing U2OS-lac-O cells in Prometaphase (PM) and Interphase (I), transiently expressing Lac-I-EGFP-CENP-U deletions (with 58-114, 1-57 and 1-114 aa). Cells were immunostained for DAPI, PLK1 and CEP135 (centrosome signal, white arrow). EGFP represents CENP-U localized at ectopic location on chromatin. White dotted circle highlights ectopic location of CENP-U and respective PLK1 signal. (**F**) Representative images showing U2OS-lac-O cells in Prometaphase (PM) and Interphase (I), transiently expressing Lac-I-EGFP-CENP-U deletions (with 86-114 and 58-85 aa). Cells were immunostained for DAPI, PLK1 and CEP135 (centrosome signal, white arrow). EGFP represents CENP-U localized at ectopic location on chromatin. White dotted circle highlights ectopic location of CENP-U and respective PLK1 signal. (**G**) Table summarizing the PLK1 ectopic localization, + (positive) or – (negative) when Lac-I-CENP-U-EGFP FL or deletions (B-D) were expressed at ectopic location on chromatin. (**H**) Depletion of endogenous CENP-OPQRU complex by CENP-OQU RNAi in HeLa cells was monitored by measuring the kinetochore levels of CENP-O by immunofluorescence analysis. Scale bar represents 5 μm. (**I**) Dot plots representing control normalized PLK1/CREST or CENP-O/CREST kinetochore intensity ratios in control cells or in cells treated for CENP-OQU RNAi. Red lines indicate median with interquartile range. A total of 764-774 kinetochores per each condition were scored. (**J**) Plot of PLK1 versus CENP-O kinetochore intensities from cells in (H-I). (**K**) Total cell lysates of cells from Figure 5 were processed for western blotting analysis of BUB1, BUBR1, and Tubulin (loading control). (**L**) Anti-GFP western blots of total lysates of cells expressing the three indicated EGFP-CENP-U variants and treated like in Figure 6D.

Figure S7 ***Additional SEC and biochemical experiments*** **(associated with Figure 4)**

(**A**) Elution profile and SDS-PAGE analysis of SEC runs on a Superdex 200 increase 5/150 column of CENP-OPQUR (5 μM; black curves) or CENP-OPQU$^{\Delta 1\text{-}114}$R (5 μM, red curves) and MBP-PLK1^{PBD} (5 μM) with (black and red curves continuous line) or without (dashed line black and red curves) the four mitotic kinases CDK1:Cyclin B, PLK1, Aurora B and BUB1. In-gel fluorescence of MBP-PLK1^{PBD} in the SEC fractions analyzed by SDS-PAGE is also shown. Orange arrows highlight MBP-PLK1^{PBD} in each gel. (**B**) Phosphorylation state of the input proteins of the SEC experiment from (4D) was assessed by SDS-PAGE followed by Coomassie or Pro-Q™ Diamond staining. (**C**) Elution profile from analytical SEC using a Superdex 200 increase 5/150 column, and subsequent SDS-PAGE analysis of mixtures of MBP-CENP-U$^{58\text{-}114}$ (5 μM) with MBP-PLK1^{PBD} (5 μM) in presence or absence of the kinases PLK1 and/or CDK1:Cyclin B. In these reactions, wild type MBP-CENP-U$^{58\text{-}114}$ and the mutant constructs were incubated with CDK1 as a priming kinase, and then separated from CDK1 by size-exclusion chromatography. Next, the CDK1-phosphorylated samples were treated with priming PLK1 in presence of 5 μM RO-3306 (to inhibit any residual CDK1 activity), before addition of MBP-PLK1^{PBD} and SEC analysis. Orange arrows highlight MBP-PLK1^{PBD} in each gel. The SDS-PAGE and elution profile for the CDK1+PLK1 condition are also displayed in Figure 6C. (**D**) Primary data and fitting residuals for the AUC of MBP-PLK1^{210A} (left) and MBP-PLK1^{210A} + CENP-OPQUR (right) shown in Figure 7A.

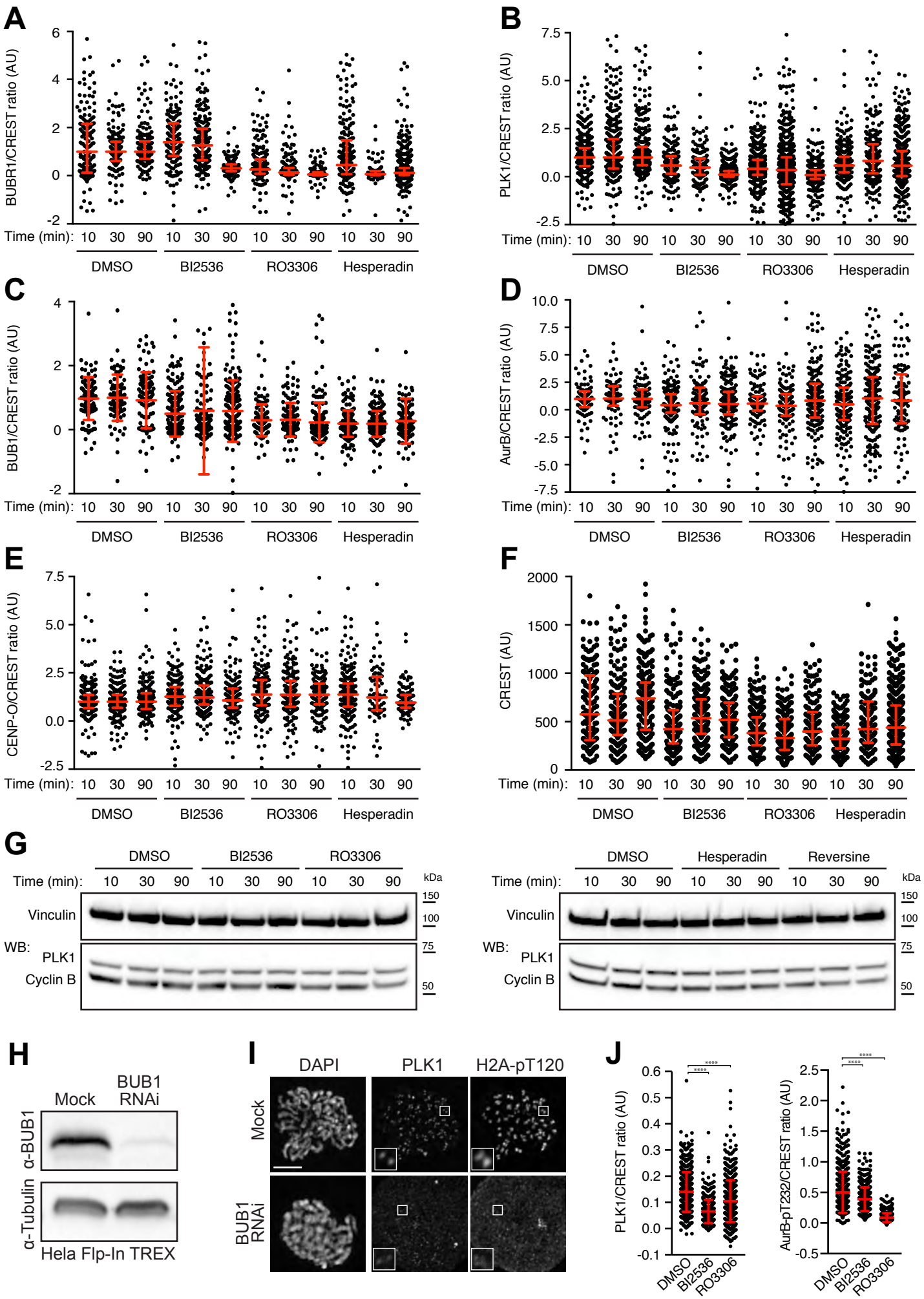

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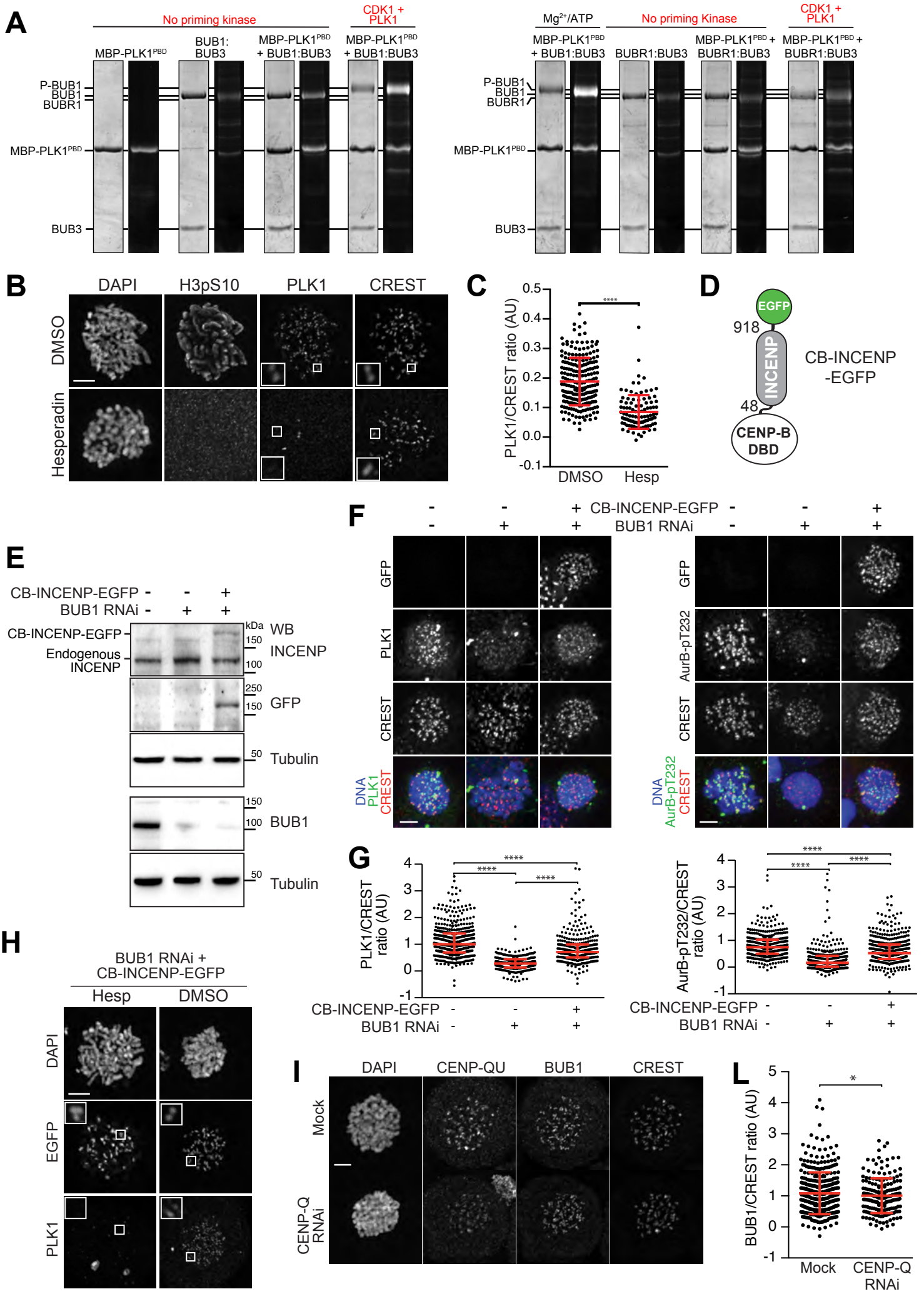
Figure S1



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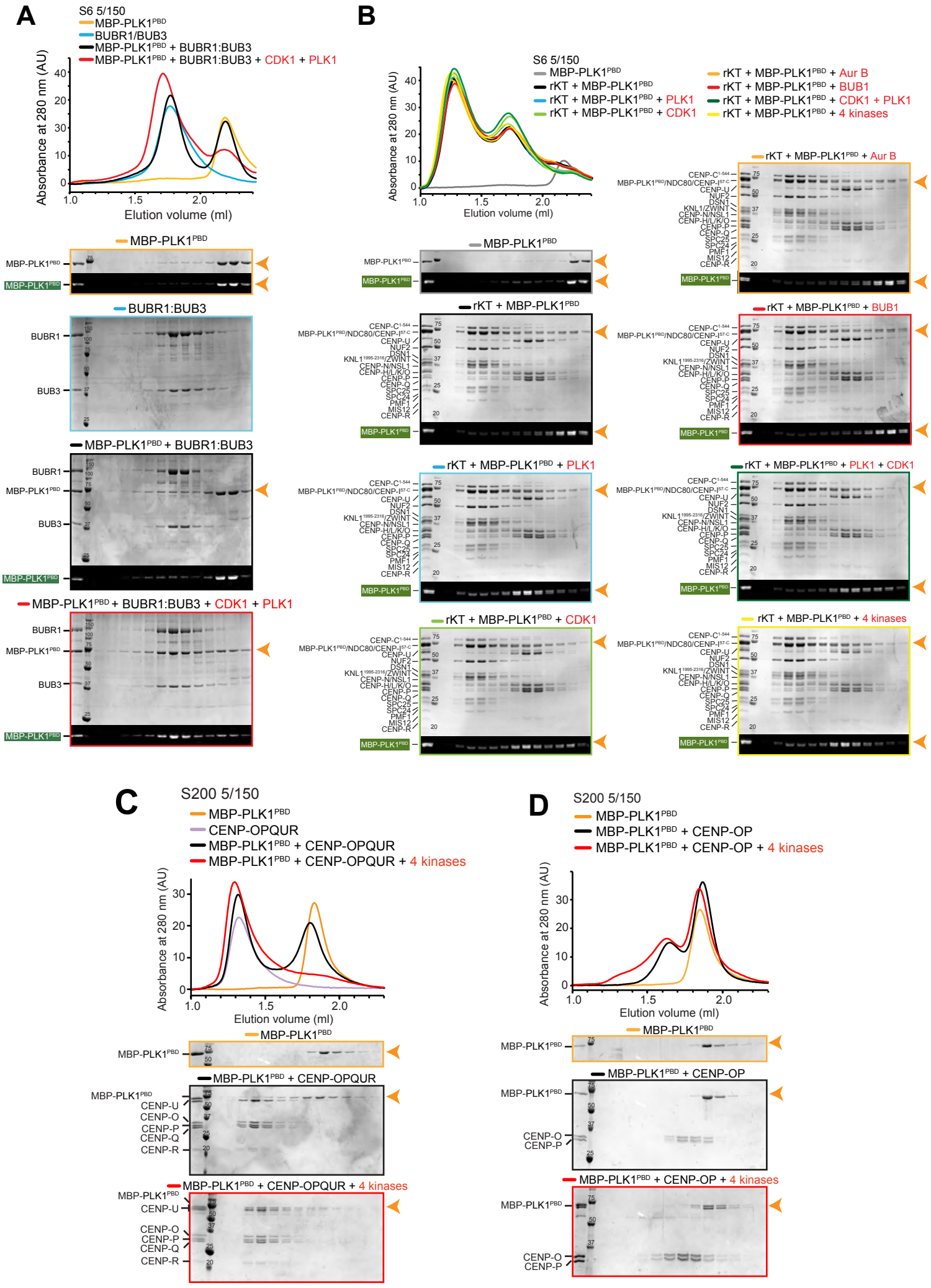

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Figure S3

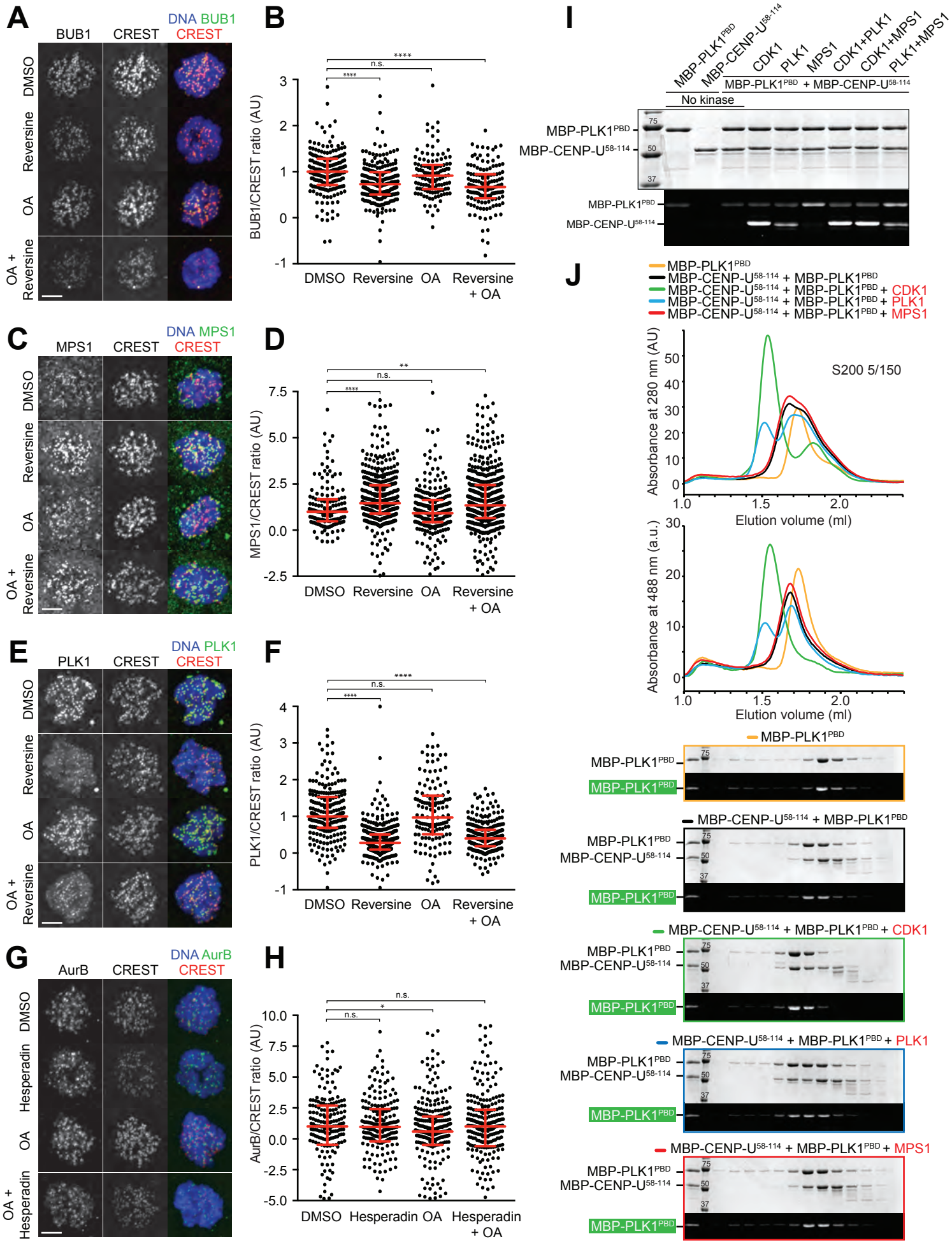

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Figure S4

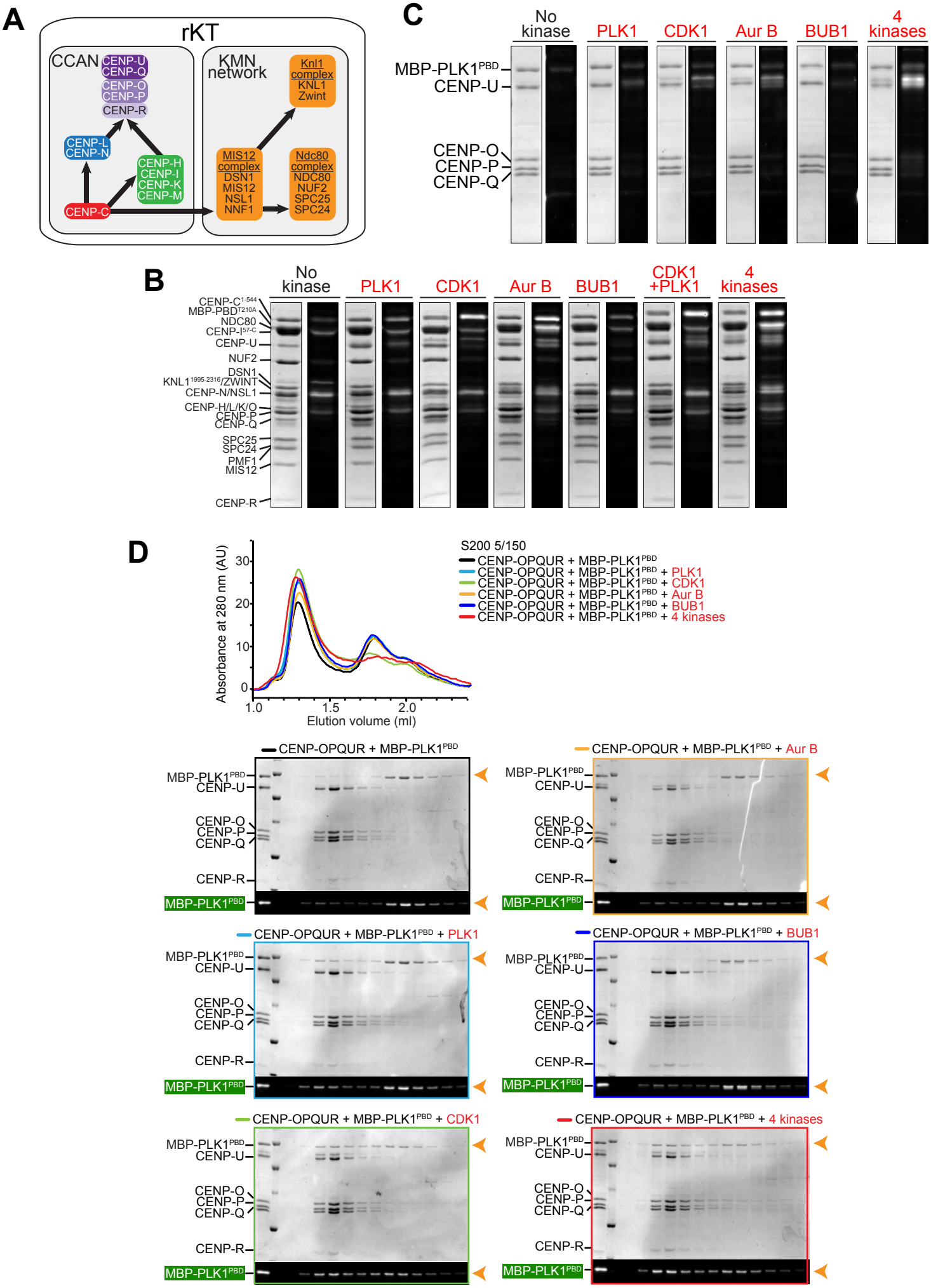

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Figure S5

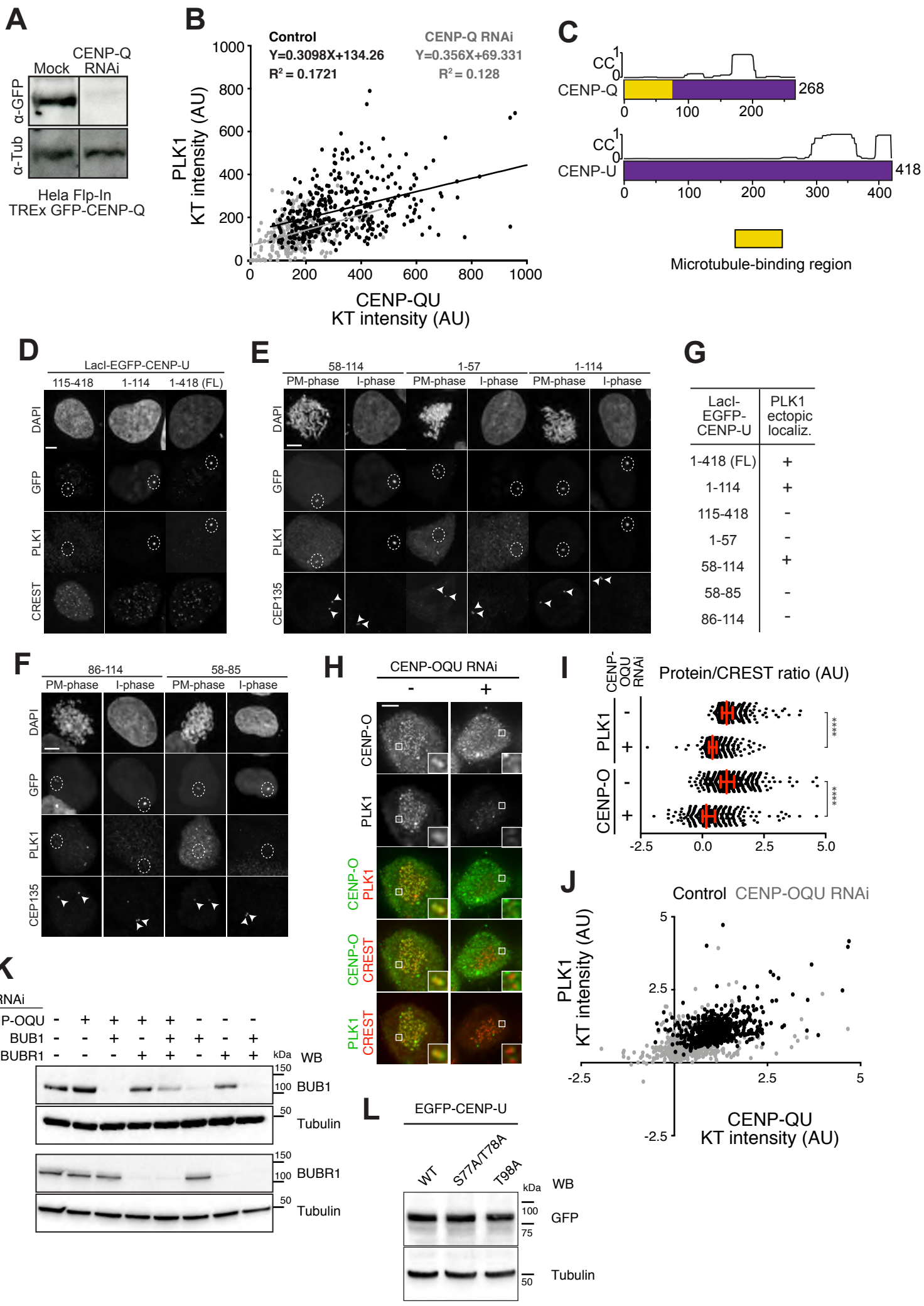

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Figure S6

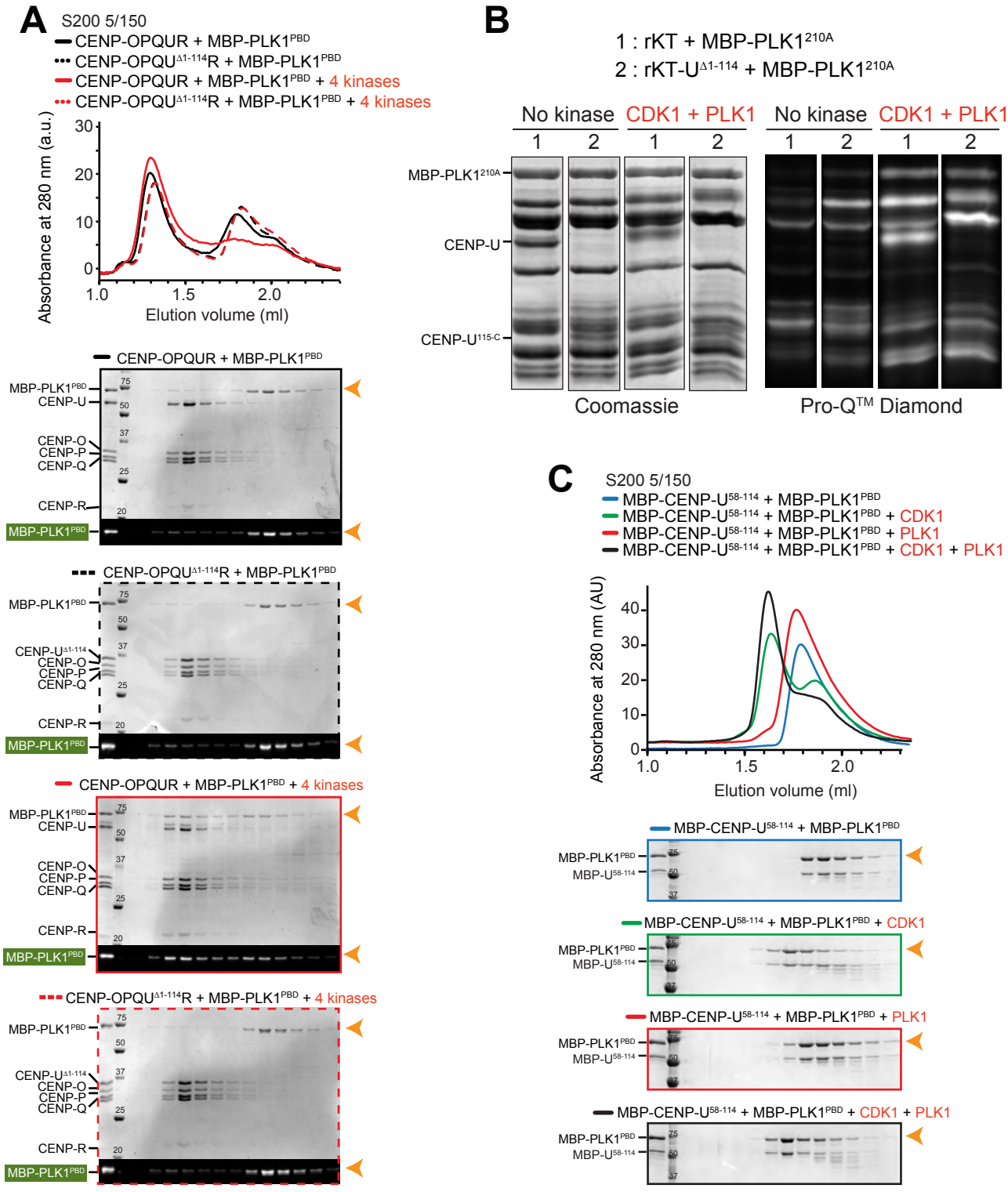

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Figure S7