Differential requirements for the CENP-O complex reveal parallel PLK1 kinetochore recruitment pathways

Alexandra L. Nguyen[a], Marie Diane Fadel[a,b], and Iain M. Cheeseman[a,b,*]
[a]Whitehead Institute for Biomedical Research, Cambridge, MA 02142; [b]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

ABSTRACT **Similar to other core biological processes, the vast majority of cell division components are essential for viability across human cell lines. However, recent genome-wide screens have identified a number of proteins that exhibit cell line–specific essentiality. Defining the behaviors of these proteins is critical to our understanding of complex biological processes. Here, we harness differential essentiality to reveal the contributions of the four-subunit centromere-localized CENP-O complex, whose precise function has been difficult to define. Our results support a model in which the CENP-O complex and BUB1 act in parallel pathways to recruit a threshold level of PLK1 to mitotic kinetochores, ensuring accurate chromosome segregation. We demonstrate that targeted changes to either pathway sensitizes cells to the loss of the other component, resulting in cell-state dependent requirements. This approach also highlights the advantage of comparing phenotypes across diverse cell lines to define critical functional contributions and behaviors that could be exploited for the targeted treatment of disease.**

Monitoring Editor
Kerry Bloom
University of North Carolina, Chapel Hill

Received: Nov 30, 2020
Revised: Feb 2, 2021
Accepted: Feb 9, 2021

INTRODUCTION

A fundamental assumption for much of the research concerning core biological processes is that the conserved players that direct these processes will exhibit similar functional requirements across organisms, let alone between cell types within a given species. However, not all proteins conform to this behavior, making the identification and analysis of molecular factors with varying requirements critical to our understanding of complex cellular biology. During eukaryotic cell division, chromosomal DNA is segregated equally between daughter cells following a tightly regulated and stereotypical choreography of chromosome capture, alignment, and distribution. The key molecular players that direct chromosome segregation, including the components of the macromolecular kinetochore structure that mediates chromosome–microtubule interactions, are conserved across most eukaryotes and are essential for cellular viability (Cheeseman and Desai, 2008). Interestingly, our recent work and the results from genome-wide screens (McKinley *et al.*, 2015; Wang *et al.*, 2015; McKinley and Cheeseman, 2017; Broad, 2020) indicate that the requirement for the centromere-localized CENP-O complex varies between human cell lines. Here, we sought to exploit this cell line–specific essentiality to define the basis for these differences between cell types and the role for this complex.

The CENP-O complex is a four-subunit interdependent protein assembly, comprised of CENP-O, CENP-P, CENP-Q, and CENP-U, that localizes constitutively to centromeric DNA as part of the larger constitutive centromere-associated network (CCAN), which collectively provides the base for kinetochore assembly (Hara and Fukagawa, 2017). The viability of many human tissue culture cell lines in the absence of the CENP-O complex is in stark contrast to other CCAN components, where perturbation results in severe mitotic defects and lethality (McKinley *et al.*, 2015). Prior work has proposed diverse functions for the CENP-O complex, including directly promoting kinetochore–microtubule attachments, promoting sister chromatid cohesion, or functioning as a scaffold for Polo-like kinase

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E20-11-0751) on February 17, 2021.

The authors declare that they have no conflicts of interest.

Author contributions: A.L.N. and I.C. designed the experiments. A.L.N. conducted the experiments. A.L.N. and M.D.F. analyzed the data. A.L.N. and I.C. wrote and edited the manuscript.

*Address correspondence to: Iain M. Cheeseman (icheese@wi.mit.edu).

Abbreviation used: CCAN, constitutive centromere-associated network.

1 (PLK1) recruitment to kinetochores (Minoshima *et al.*, 2005; Foltz *et al.*, 2006; Kang *et al.*, 2006, 2011; Pesenti *et al.*, 2018). However, a lack of strong phenotypes observed for the loss of the CENP-O complex in the cell lines typically used for analyses of cell division, such as HeLa cells, has made defining the role of this complex difficult. Here, we harness the cell line–specific requirements of the CENP-O complex to define its primary functional contribution in cellular division. Based on our analysis of the CENP-O complex across multiple human cell lines, our work suggests that the primary functional contribution of the CENP-O complex is in the recruitment of PLK1 to kinetochores. Our work reveals that PLK1 recruitment occurs through parallel pathways that are governed by BUB1 and the CENP-O complex such that changes to either pathway sensitizes cells to loss of the other, resulting in cell line–specific essentiality. This finding is also supported by recent studies by Singh *et al.*, (2020), in which the authors reconstituted the recruitment of PLK1 to in vitro assembled kinetochores via BUB1 and CENP-U, and Chen *et al.*, (2021).

Together, our work identifies the source for the differential requirement of the CENP-O complex across cell lines. Importantly, this approach also highlights the advantage of comparing differential requirements and phenotypes across diverse cell lines and cell types, particularly for defining the function of previously difficult to characterize proteins. The investigation of cell line–specific protein essentialities will also prove valuable in pinpointing disease-specific vulnerabilities, allowing the identification of directed diagnostic and therapeutic targets.

RESULTS

The CENP-O complex exhibits differential requirements in human cell lines

Despite the conservation of the four-subunit CENP-O complex across diverse eukaryotes, our previous work found that eliminating CENP-O from human HeLa cell lines did not result in substantial defects in chromosome segregation or viability (McKinley *et al.*, 2015; McKinley and Cheeseman, 2017). Intriguingly, recent genome-wide functional screens found that the CENP-O complex is not required in most human cell lines, but also identified multiple cell lines that display a strict requirement for the CENP-O complex (Wang *et al.*, 2015; Meyers *et al.*, 2017; Broad, 2020). To define the basis for the cell line–specific requirements for the CENP-O complex, we used a Cas9-inducible gene targeting strategy (McKinley *et al.*, 2015; McKinley and Cheeseman, 2017) in multiple human cell lines. As the CENP-O complex subunits display interdependent localization (Hori *et al.*, 2008) and genome-wide functional analyses have revealed similar behaviors for each subunit (Wang *et al.*, 2015; Meyers *et al.*, 2017; Broad, 2020), for these experiments we targeted two representative CENP-O complex subunits, CENP-O and CENP-U. For our initial analysis, we compared HeLa cells, a cervical cancer cell line that our previous work found is insensitive to the loss of CENP-O; the diploid and nontransformed RPE-1 cell line; and K-562 cells, a leukemogenic cell line that exhibits proliferation defects upon gene targeting of CENP-O complex subunits based on genome-wide screens (Wang *et al.*, 2015).

We first defined the phenotypes resulting from the inducible knockout (iKO) of CENP-O or CENP-U. Due to the nature of the inducible knockout system, a subset of Cas9 cleavage events will be repaired in a manner that retains the open reading frame resulting in a mixed population of cells. Importantly, we did not observe a difference in the proportion of cells in which the CENP-O complex was eliminated across HeLa, RPE-1, or K-562 cell line backgrounds as determined by immunofluorescence analysis with antibodies specific for CENP-O/P (Supplemental Figure S1A; McKinley *et al.*, 2015), allowing us to compare behaviors between cell lines. Loss of CENP-O or CENP-U did not significantly affect chromosome alignment in HeLa or RPE-1 cells, indicating that the lack of a strong phenotype is unrelated to p53 status. In contrast, knockout of either protein resulted in dramatic mitotic defects in K-562 cells, with 39% of CENP-O iKO and 30% of CENP-U iKO cells exhibiting misaligned chromosomes, as defined by the presence of at least one off-axis chromosome, compared with 8% of control cells (Figure 1, A and B, and Supplemental Figure S1, B and C). Despite the presence of misaligned chromosomes, K-562 CENP-O and CENP-U iKO cells failed to arrest in metaphase, resulting in a proportion of cells with anaphase chromosome segregation defects, including lagging chromosomes and anaphase bridges (Figure 1, C and D, and Supplemental Figure S1, D and E). We note that, despite an increase in anaphase phenotypes upon CENP-O/U loss, K-562 cells exhibited higher proportions of anaphase defects independent of CENP-O/U status. Together, these data highlight core differences in the sensitivity of different human cell lines to the loss of the CENP-O complex, and demonstrate that this complex contributes to chromosome alignment and segregation in specific human cell lines.

The CENP-O complex promotes PLK1 recruitment to kinetochores

The CENP-O complex localizes constitutively to centromeres as part of the inner kinetochore CCAN and has been proposed to perform diverse roles, including functioning as a scaffold for PLK1 recruitment to kinetochores, directly promoting kinetochore–microtubule attachments, and promoting sister chromatid cohesion (Minoshima *et al.*, 2005; Foltz *et al.*, 2006; Kang *et al.*, 2006; Elowe *et al.*, 2007; Kang *et al.*, 2011; Pesenti *et al.*, 2018). Each of these proposed functions is considered to be an essential process for all dividing cells, raising the question of why the CENP-O complex proteins would exhibit cell line–specific requirements. Prior work has focused on the functional analysis of the CENP-O complex in human cell lines where this complex is not required for viability, such as HeLa cells. Because loss of the CENP-O complex in K-562 cells results in significant increase in mitotic defects, this phenotype provides the opportunity to define the critical contributions of the CENP-O complex under conditions where it is required for cell division. The differences in CENP-O complex requirements across cell lines likely reflect underlying genetic or physiological susceptibilities that cause a given cell line to be predisposed to CENP-O complex loss. In assessing differences between cell lines, we observed a striking difference in the levels of kinetochore-localized PLK1, with reduced PLK1 levels in K-562 cells compared with HeLa and RPE-1 cells (Figure 2, A and B, and Supplemental Figure S2, A and B). For these experiments, we normalized PLK1 intensity relative to the levels of NDC80, a core component of the outer kinetochore, to ensure these differences did not reflect variations in kinetochore size between cell lines. Importantly, the difference in PLK1 levels was specific to the kinetochore-bound population of PLK1, as centrosomal and spindle midzone-localized PLK1 were not notably different between cell lines (Supplemental Figure S2, C and D). In addition, total PLK1 protein levels were not significantly different between HeLa and K-562 cells, independent of CENP-O status (Supplemental Figure S2E), suggesting that the reduced levels of kinetochore-localized PLK1 reflect differences in the recruitment of the kinase to kinetochores.

We next sought to test whether the varying levels of kinetochore-localized PLK1 could underlie the differential requirements for the CENP-O complex between cell lines. CENP-U binds directly to PLK1 and this binding has been proposed to promote PLK1 localization to

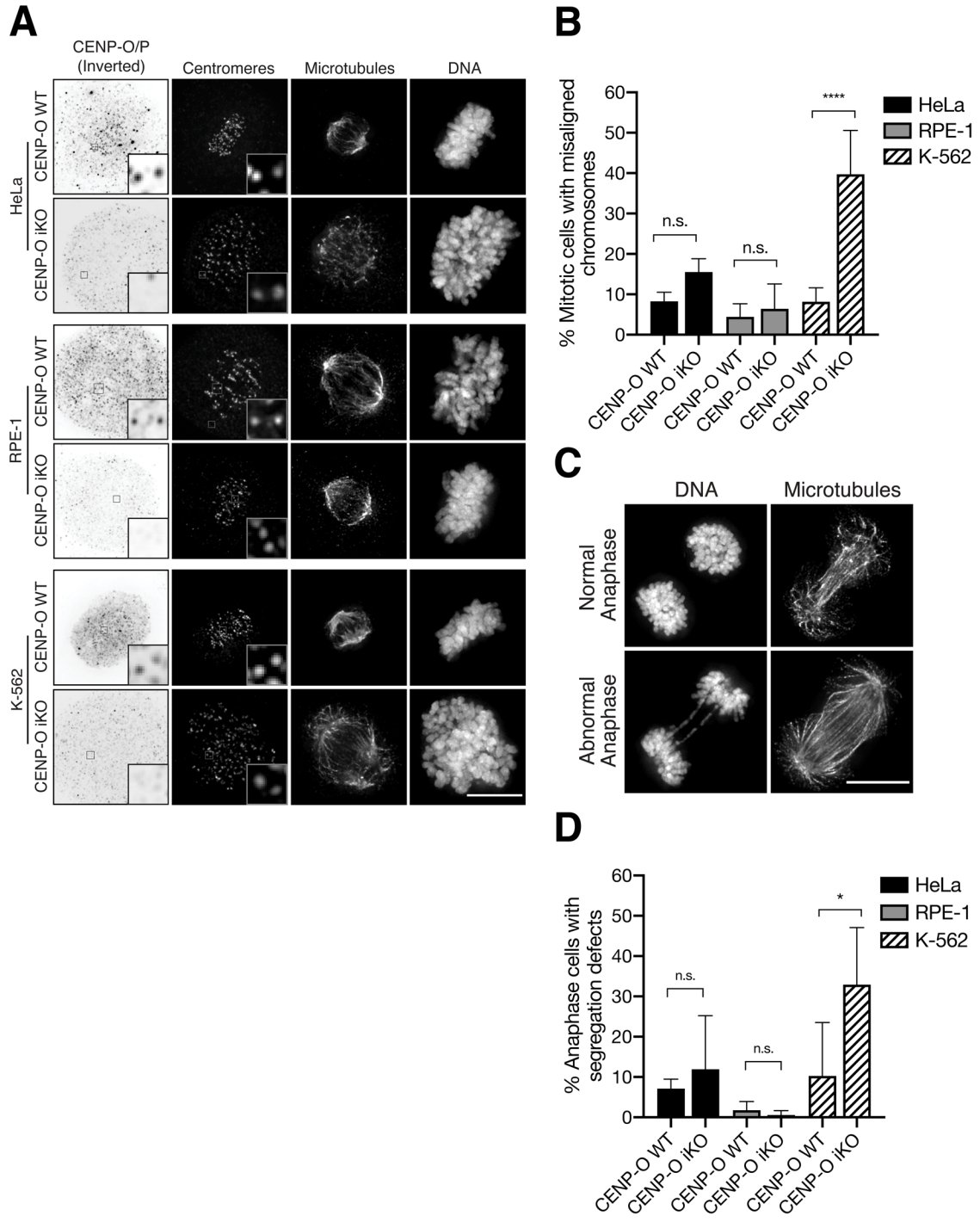

FIGURE 1: The CENP-O complex exhibits differential requirements in human cell lines. (A) Representative Z-projected immunofluorescence images of metaphase cells from CENP-O inducible knockout (iKO) HeLa, RPE-1, and K-562 cell lines. Images show anti-CENP-O/P antibodies (inverted), centromeres (ACA), microtubules (DM1α), and DNA (Hoechst). Boxes indicate areas of optical zoom. (B) Percent mitotic cells with misaligned chromosomes after inducible knockout of CENP-O for 5 d, quantified from A. *n* = approximately 300 cells per condition, across three experimental replicates. (C) Representative Z-projected immunofluorescence images of anaphase cells from CENP-O inducible knockout HeLa, RPE-1, and K-562 cell lines. Spindle (DM1α), DNA (Hoechst). (D) Quantification of anaphase cells with defects including chromosome bridges and lagging chromosomes from C. Representative anaphase cells are from CENP-U control and CENP-U iKO K-562 cell lines. *n* = approximately 100 cells per condition across three experimental replicates. Error bars indicate SD. One-way ANOVA was performed (* = 0.0366, **** = <0.001). Scale bars, 10 μM. See also Supplemental Figure S1.

kinetochores (Kang *et al.*, 2006; Lee *et al.*, 2008; Kang *et al.*, 2011; Park *et al.*, 2015). Based on this predicted function, we hypothesized that a threshold level of kinetochore-localized PLK1 is required for accurate chromosome segregation, and that cell lines with reduced PLK1 would be sensitized to CENP-O/U depletion. Consistent with this hypothesis, we found that eliminating CENP-O or CENP-U

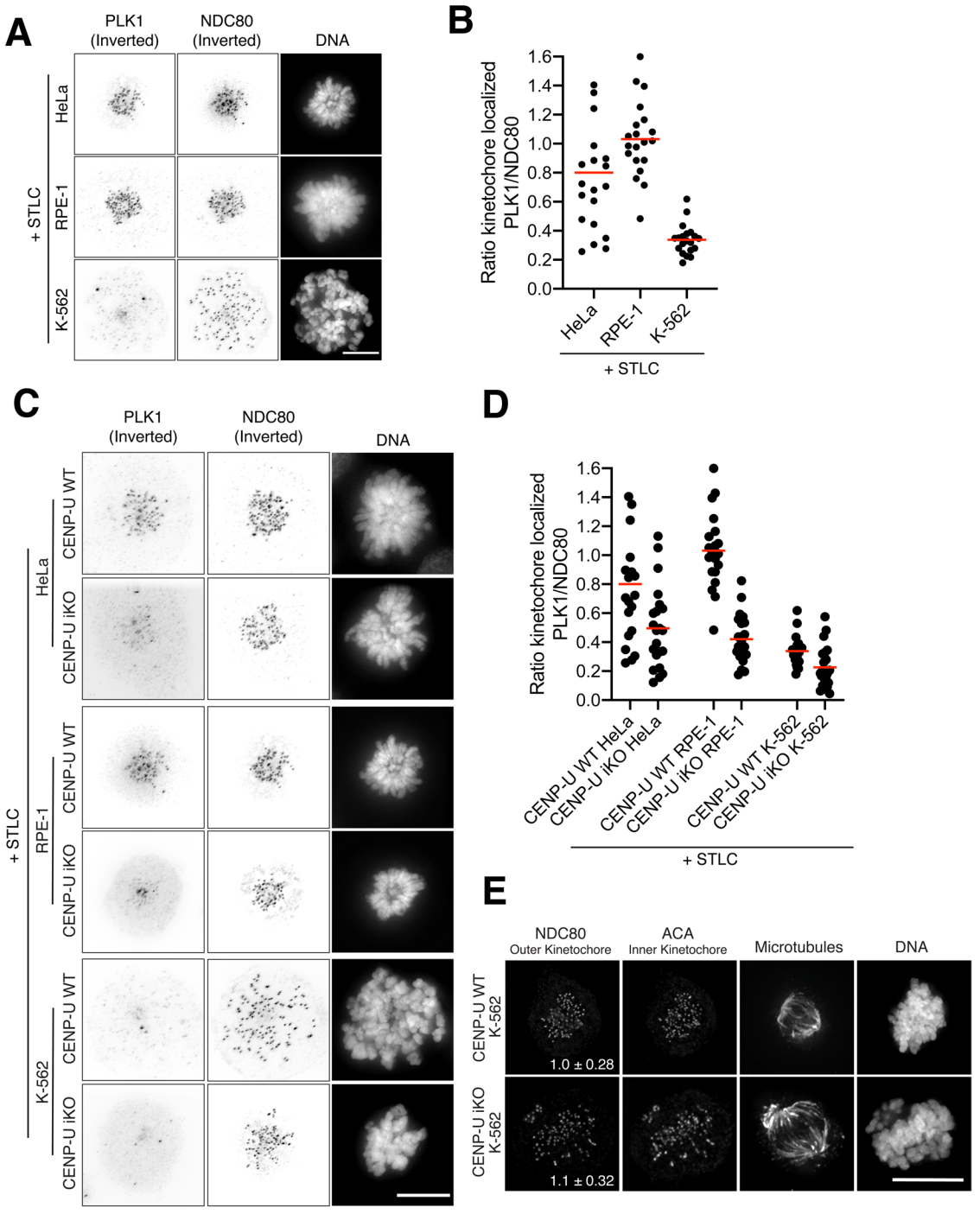

FIGURE 2: The CENP-O complex recruits PLK1 to mitotic kinetochores. (A) Representative Z-projected immunofluorescence images of STLC-arrested metaphase cells from HeLa, RPE-1, and K-562 cell lines. Images show anti-PLK1 antibodies (inverted), NDC80 (inverted), and DNA (Hoechst). To ensure a comparison of PLK1 levels at similar stages of mitosis, cells were synchronized via incubation in the Kif11 inhibitor STLC overnight before fixation. (B) Relative pixel intensity of kinetochore-localized PLK1, normalized to NDC80 for each cell line, from A. Each data point represents a single cell. $n = 20$ cells per group. Red bars indicate mean. (C) Representative Z-projected immunofluorescence images of STLC-arrested metaphase cells from CENP-U inducible knockout HeLa, RPE-1, and K-562 cell lines. Images show anti-PLK1 antibodies (inverted), NDC80 (inverted), and DNA (Hoechst). (D) Relative pixel intensity of kinetochore-localized PLK1, normalized to NDC80 for each cell line, from C. Each data point represents a single cell. Red bars indicate mean. n = approximately 20 cells per group. Control cell line data is the same as that represented in A and B. (E) Representative Z-projected immunofluorescence images of mitotic cells from the CENP-U inducible knockout K-562 cell line after inducible knockout of CENP-U for 5 d showing NDC80, anti-centromere antibodies (ACA), microtubules (DM1α), and DNA (Hoechst). Inset ratios represent the relative pixel intensity of kinetochore-localized NDC80 ± SD, normalized to control cells. n = approximately 40 cells per group across two experimental replicates. Student's t test was performed with no significant difference observed. Scale bars, 10 μM. See also Supplemental Figures S2 and S3.

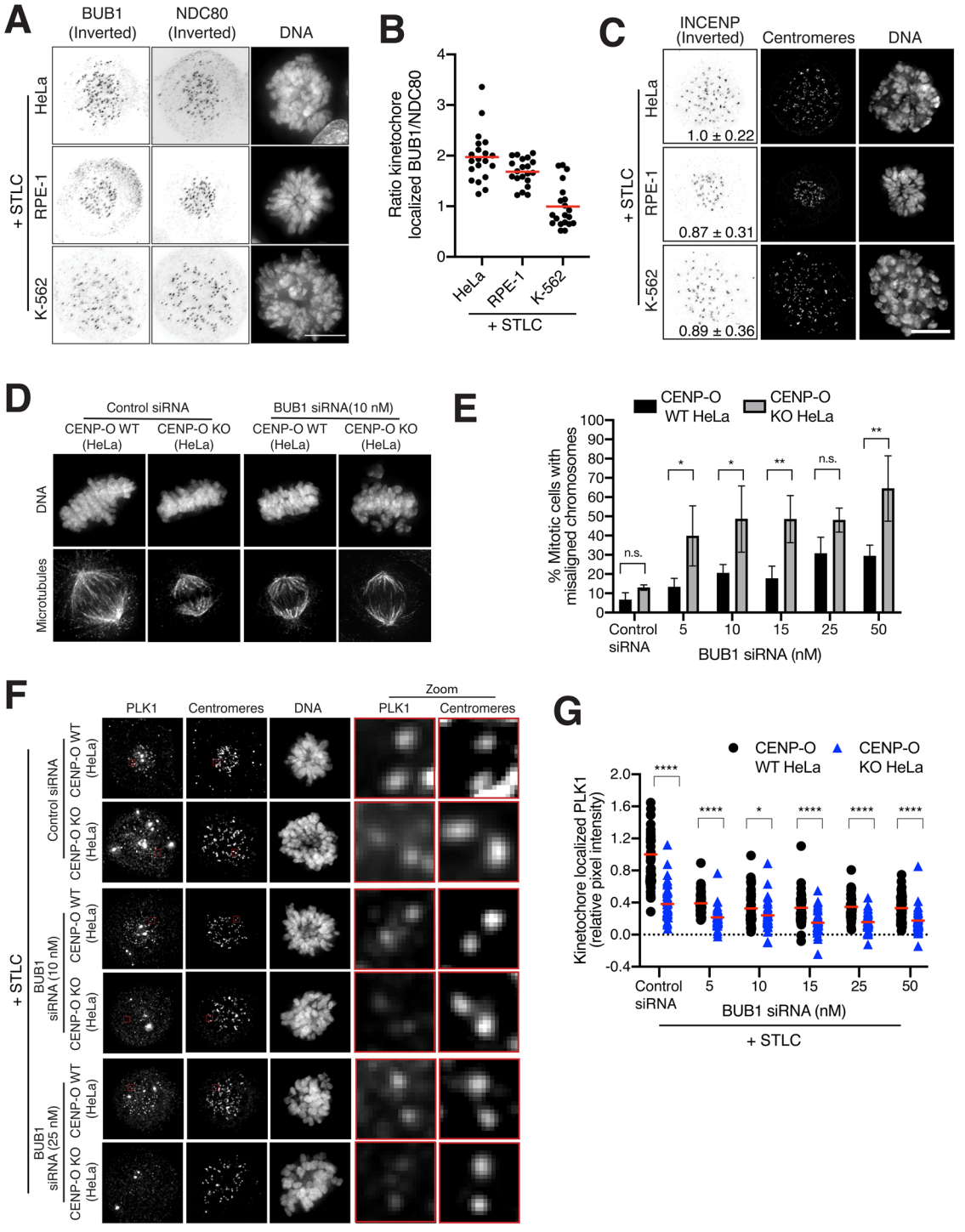

FIGURE 3: Reducing BUB1 expression sensitizes cells to the loss of the CENP-O complex. (A) Representative Z-projected immunofluorescence images of STLC-arrested metaphase cells from HeLa, RPE-1, and K-562 cell lines showing anti-BUB1 antibodies (inverted), NDC80 (inverted), and DNA (Hoechst). To ensure a comparison of protein levels at similar stages of mitosis, cells were synchronized via incubation in the Kif11 inhibitor STLC overnight before fixation. (B) Relative pixel intensity of kinetochore-localized BUB1, normalized to NDC80 for each cell line, from A. Each data point represents a single cell. $n = 20$ cells per group. Red bars indicate mean. (C) Representative Z-projected immunofluorescence images of STLC-arrested metaphase cells from HeLa, RPE-1, and K-562 cell lines showing anti-INCENP (inverted), centromeres (ACA), and DNA (Hoechst). Inset ratios represent the relative pixel intensity of kinetochore-localized INCENP ± SD, normalized to HeLa. n = approximately 30 cells per group across two experimental replicates. One-way ANOVA was performed with no significant difference observed. (D) Z-projected immunofluorescence images of metaphase cells of the indicated cell lines incubated in the presence of control siRNA or 10 nM BUB1 siRNA showing microtubules (DM1α) and DNA (Hoechst). HeLa CENP-O WT and stable CENP-O knockout (KO) cells were incubated in the presence of the indicated concentrations BUB1 siRNA or nontargeting control for 48 h

resulted in a significant reduction in kinetochore-localized PLK1 in all the cell lines tested compared with controls from the corresponding cell line (Figure 2, C and D, and Supplemental Figure S3, A–D). CENP-O/U depletion resulted in reduced PLK1 kinetochore localization in all cell lines, but the level of PLK1 at kinetochores was significantly lower in K-562 CENP-O and CENP-U iKO cells compared with HeLa CENP-O/U iKO or RPE-1 CENP-O/U iKO cells. Although PLK1 levels were variable across experiments, we observed a consistent trend in which a large proportion of K-562 cells exhibited substantially lower PLK1 levels than either HeLa or RPE-1 cells, with a greater spread observed across the population. In contrast to PLK1 localization, the localization of the outer kinetochore component NDC80 was not affected by CENP-U loss and was independent of cell line (Figure 2E). This indicates that the mitotic defects observed upon the loss of the CENP-O complex in K-562 cells are not the result of general kinetochore assembly defects. These results support a model in which the CENP-O complex promotes the recruitment of PLK1 to mitotic kinetochores.

Although eliminating CENP-O or CENP-U results in a reduction in kinetochore-localized PLK1, PLK1 localization is not lost completely suggesting that additional kinetochore-localized PLK1 binding partners contribute to its recruitment (Figure 2, C and D, and Supplemental Figure S3, A–C; Kang *et al.*, 2006, 2011; Lee *et al.*, 2008). In addition to CENP-U (Kang *et al.*, 2006; Lee *et al.*, 2008), multiple kinetochore-localized proteins have been proposed to serve as PLK1 binding factors, including BUB1 and INCENP (Goto *et al.*, 2006; Kang *et al.*, 2006; Qi *et al.*, 2006). Therefore, we next investigated whether these alternate PLK1 recruitment pathways were altered in K-562 cells, creating a synthetic lethal-like relationship for the CENP-O complex. Interestingly, we found that BUB1 levels, but not INCENP levels, were significantly lower at kinetochores in K-562 cells when compared with HeLa or RPE-1 (Figure 3, A–C, and Supplemental Figure S4, A and B). Consistently, total BUB1 protein levels also appeared mildly reduced in K-562 cells, as compared with HeLa (Supplemental Figure S2E), suggesting that the differential expression of kinetochore-localized binding partners for PLK1 could underlie the cell type–specific requirement for the CENP-O complex. Interestingly, total BUB1 protein levels were further reduced in the absence of the CENP-O complex in K-562 cells, suggesting a possible feedback mechanism or outer kinetochore dependence on the CENP-O complex in this cell line (Supplemental Figure S2E).

The CENP-O complex and BUB1 collaborate to recruit PLK1 to mitotic kinetochores

Based on the data described above, we hypothesized that BUB1 and the CENP-O complex act in parallel to recruit PLK1 to mitotic kinetochores such that cell lines with reduced kinetochore-localized BUB1 would have an increased requirement for the CENP-O complex. To test this model, we sought to sensitize cell lines in which the CENP-O complex is otherwise dispensable by generating varying levels of BUB1 using partial RNAi-based depletion in HeLa cells, a cell line that is not normally sensitive to CENP-O loss. Strikingly, CENP-O knockout HeLa cells were hypersensitive to the reduction in BUB1 levels, with concentrations as low as 5 nM BUB1 siRNA resulting in a significant increase in chromosome misalignment (Figure 3, D and E). This phenotype is in stark contrast to control cells, in which only a modest increase in chromosome alignment was observed at concentrations below 25 nM BUB1 siRNA. The increase in mitotic defects observed upon BUB1 depletion correlated with a dose-dependent reduction in kinetochore-localized PLK1 in both control and CENP-O KO HeLa cells, consistent with a role for BUB1 in promoting PLK1 kinetochore localization (Figure 3, F and G). Due to poor efficiency of siRNA transfection in K-562 cells using standard transfection techniques, we were unable to conduct similar experiments in this cell background.

The sensitivity of CENP-O knockout HeLa cells to PLK1 loss was specific to BUB1 perturbation, as knockdown of INCENP did not result in a significant increase in chromosome misalignment in CENP-O knockout HeLa cells when compared with controls (Supplemental Figure S5, A and B). Notably, at the concentrations of INCENP siRNA employed in this study, no significant difference in kinetochore-localized PLK1 was observed between control and siRNA knockdown cells (Supplemental Figure S5, C and D). Furthermore, these effects were not the result of a deficient spindle assembly checkpoint, as partial depletion of MAD2 resulted in comparable defects in both control and CENP-O knockout HeLa cells (Supplemental Figure S5, E and F). Together, these data support a model in which BUB1 and the CENP-O complex cooperate to recruit PLK1 to mitotic kinetochores such that altering either pathway creates an increased reliance on the other pathway to ensure sufficient PLK1 localization to kinetochores.

The requirement for the CENP-O complex is dependent upon PLK1 recruitment to kinetochores

Our data support a model in which the requirement for the CENP-O complex varies depending upon the levels of kinetochore-localized BUB1 (Figure 3, D–G) due to a role for these two pathways in promoting PLK1 recruitment to mitotic kinetochores. However, BUB1 plays diverse roles in the control of cell division in addition to its ability to recruit PLK1 to kinetochores (Marchetti and Venkatachalam, 2010; Combes *et al.*, 2017). To determine whether the inability to recruit sufficient PLK1 to mitotic kinetochores underlies the synergistic phenotypes observed for the loss of CENP-O and BUB1, we generated a mutation in BUB1 (T609A) that prevents PLK1 binding without interfering with its other known activities (Qi *et al.*, 2006). We then expressed either wild-type (WT) GFP-BUB1 or the GFP-BUB1-T609A mutant in HeLa control or CENP-O knockout cells

before analysis. (E) Percent mitotic cells with misaligned chromosomes from D. Error bars indicate SD. *N* = approximately 300 cells per condition/per group, across three experimental replicates. Two-way ANOVA was performed. (5 nM) * = 0.02, (10 nM) * = 0.01, (15 nM) ** = 0.006, (50 nM) ** = 0.001. (F) Representative Z-projected immunofluorescence images of STLC-arrested metaphase cells of the indicated cell lines incubated in the presence of control siRNA and 10 nM BUB1 siRNA showing anti-PLK1 antibodies, centromeres (ACA), and DNA (Hoechst). To ensure a comparison of PLK1 levels at similar stages of mitosis, cells were synchronized via incubation in the Kif11 inhibitor STLC overnight before fixation. Boxes indicate areas of optical zoom. (G) Relative pixel intensity of kinetochore-localized PLK1 from F, normalized to control siRNA CENP-O WT HeLa. *n* = approximately 50 cells per group, across two experimental replicates. Red bars indicate the mean. Statistics represent *t* test comparing control, and CENP-O knockout PLK1 measures per concentration siRNA (* = 0.03, **** = <0.001). Scale bars, 10 μM. See also Supplemental Figures S4 and S5.

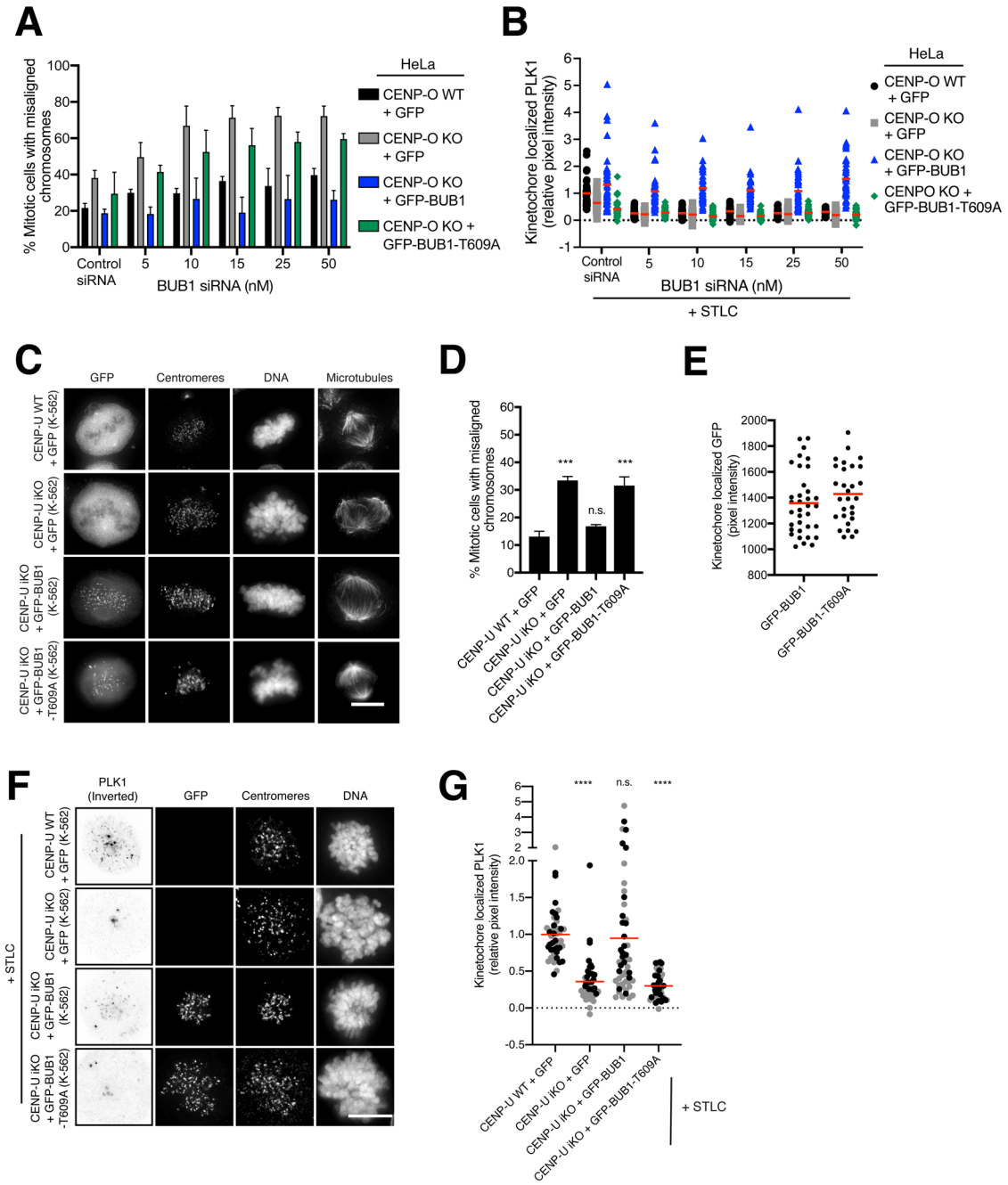

FIGURE 4: Ectopic BUB1 expression can suppress the cell line–specific requirement for the CENP-O complex. (A, B) HeLa control and stable CENP-O knockout (KO) cells expressing the indicated constructs were incubated in the presence of the indicated concentrations BUB1 siRNA or nontargeting control for 48 h before analysis. (A) Percent mitotic cells with misaligned chromosomes from the indicated cell lines after 48 h BUB1 siRNA knockdown. Error bars indicate SD. *n* = ~300 cells per condition/per group, across three experimental replicates. (B) Relative pixel intensity of kinetochore-localized PLK1 in the indicated cell lines after 48 h BUB1 siRNA knockdown followed by overnight incubation in STLC, normalized to control siRNA CENP-O WT + GFP HeLa. *n* = approximately 50 cells per group, across two experimental replicates. (C) Representative Z-projected immunofluorescence images of metaphase cells from CENP-U inducible knockout K-562 cell lines expressing the indicated constructs showing GFP, centromeres (ACA), DNA (Hoechst), and microtubules (DM1α). Fixed cells were incubated in GFP-booster to increase signal. (D) Percent mitotic cells with misaligned chromosomes after inducible knockout of CENP-U for 5 d, from C. *n* = ~250 cells per condition, across three experimental replicates. Error bars indicate SD. One-way ANOVA performed. (CENP-U iKO + GFP) *** = 0.0007; (CENP-U iKO + GFP-BUB1-T609A) *** = 0.0002. (E) Pixel intensity of kinetochore-localized GFP-BUB1 and GFP-BUB1-T609A in CENP-U KO K-562 cells. (F) Representative Z-projected immunofluorescence images of STLC-arrested metaphase cells from CENP-U inducible knockout K-562 cell lines expressing the indicated constructs showing anti-PLK1 antibodies (inverted), GFP, DNA (Hoechst), and microtubules (DM1α). To ensure a comparison of PLK1 levels at similar stages of mitosis, cells were synchronized via incubation in the Kif11 inhibitor STLC overnight before

(Figure 3, D–G). Expression of an RNAi-resistant version of GFP-BUB1 was sufficient to rescue the chromosome alignment defects in both CENP-O WT and CENP-O KO cells across diverse BUB1 siRNA concentrations (Figure 4A). The observed rescue of mitotic defects correlated with a significant increase in kinetochore-localized PLK1 upon GFP-BUB1 expression (Figure 4B). In contrast, expression of the PLK1 binding-deficient BUB1 mutant (T609A) failed to rescue the chromosome alignment or restore Plk1 localization to mitotic kinetochores (Figure 4, A and B). These data highlight the functional requirement for BUB1 to associate with and recruit PLK1 to kinetochores in creating the synergistic phenotypes with the CENP-O complex.

Defects in PLK1–kinetochore recruitment underlie the chromosome segregation defects in CENP-O knockout cells

We hypothesize that BUB1 and the CENP-O complex collaborate to ensure a sufficient quantity of PLK1 localizes to mitotic kinetochores. If cells fail to achieve this threshold level of PLK1, they will be unable to accurately segregate their chromosomes. In support of this model, we are able to sensitize cells that are normally resistant to CENP-O complex loss simply by reducing BUB1 levels via siRNA-mediated knockdown (Figure 3, D and E). To further test the model that PLK1 recruitment underlies the cell line–specific requirement of the CENP-O complex, we next sought to rescue the defects observed upon CENP-O/U loss in K-562 cells by increasing BUB1 levels. To this end, we tested whether ectopic expression of GFP-BUB1 could rescue the mitotic defects observed in CENP-U iKO K-562 cells. Strikingly, stable expression of GFP-BUB1 was sufficient to rescue the mitotic defects observed upon inducible knockout of CENP-U, with levels of chromosome misalignment comparable to control cells (Figure 4, C–E). Similarly, ectopic expression of GFP-BUB1 resulted in a significant increase in PLK1 kinetochore localization in CENP-U iKO K-562 cells, comparable to those observed in control cells (Figure 4, F and G). In contrast, expression of GFP-BUB-T609A, which is defective in PLK1 binding, was unable to rescue chromosome alignment or PLK1 localization in CENP-U iKO cells (Figure 4, C–G). Taken together, these data suggest that BUB1 and the CENP-O complex act in parallel pathways to recruit a threshold level of PLK1 to kinetochores. The presence of either pathway is sufficient to promote PLK1 function at kinetochores, but perturbations to one of these proteins generates a synthetic lethal requirement for the other to ensure a threshold level of PLK1 is maintained. Failure to recruit a minimum level of PLK1 results in severe chromosome segregation defects.

DISCUSSION

Parallel PLK1 kinetochore recruitment pathways underlie differential CENP-O complex requirements

The functional contributions of the CENP-O complex to cell division have been difficult to define, in part due to the insensitivity of many cell lines to its loss. Here, using a combination of cell biological and genetic approaches, we find that a primary functional contribution for the CENP-O complex in human cells is to recruit PLK1 to mitotic kinetochores. The role of PLK1 at mitotic kinetochores has been of great interest (Lera *et al*., 2019). However, as PLK1 maintains multiple distinct localizations, and plays diverse roles during mitosis, including in centrosome function, cytokinesis, spindle orientation, and other tasks (Colicino and Hehnly, 2018), strategies that globally inhibit PLK1 are unable to reveal the precise functions of PLK1 at kinetochores. Because the specific mechanisms of PLK1 recruitment to kinetochores have remained elusive, so have its kinetochore contributions. Importantly, our work demonstrates that PLK1 kinetochore localization is dependent upon parallel BUB1 and CENP-U–based recruitment pathways that together ensure a threshold level of PLK1 localization to kinetochores, promoting accurate chromosome alignment and segregation. Perturbations to either of these pathways result in a sensitized requirement for the remaining PLK1 binding partner. This is consistent with previous work that found that even subtle reductions in PLK1 activity can severely impact chromosome congression, indicating distinct activity thresholds are required for proper kinase function (Lera and Burkard, 2012). Additionally, recent work from Singh *et al*. and Chen *et al*. further supports a model in which BUB1 and CENP-U serve as the primary recruitment pathways for PLK1 to mitotic kinetochores (Singh *et al*., 2020; Chen *et al*., 2021). It is important to note that PLK1 localization to kinetochores is dynamic and occurs at various stages of the cell cycle (Barr *et al*., 2004). Although our work and that from Singh *et al*. and Chen *et al*. provide strong evidence for BUB1 and CENP-U acting as the primary nodes for PLK1 recruitment to kinetochores in early mitosis, the extent to which other PLK1 binding partners contribute to PLK1 kinetochore localization throughout the cell cycle is an important question for future research.

This work also highlights an important role for PLK1 recruitment to kinetochores in mitotic chromosome alignment and segregation. Specifically, in situations with reduced PLK1 localization to kinetochores, chromosomes are unable to align at the metaphase plate and display defective segregation in anaphase (Figures 1, A–D and 2, C and D, and Supplemental Figures S1, B–E, and S3, A–D). These results are consistent with prior work in which the specific inactivation of inner kinetochore-localized PLK1 disrupted chromosome alignment and segregation (Lera *et al*., 2016), and provide evidence for CENP-U being the primary inner kinetochore PLK1 binding partner. Whether the CENP-O complex maintains functions outside of PLK1 kinetochore recruitment and if these additional roles also contribute to chromosome alignment remain an important area of investigation. Interestingly, we find that the differential requirement exhibited by the CENP-O complex in K-562 cells, when compared with HeLa and RPE-1 cell lines, reflects differences in the BUB1-PLK1 recruitment pathway. It is important to note that K-562 cells, independent of the presence of the CENP-O complex, exhibited higher rates of chromosome segregation defects when compared with HeLa or RPE-1 cell lines. We hypothesize that this difference reflects a weakened checkpoint response due to reduced localization of BUB1 to kinetochores. The aberrant expression of the oncogenic Bcr-Abl fusion, a marker of chronic myeloid leukemogenic cell lines such as K-562, has been shown to result in the down-regulation of multiple mitotic checkpoint genes, including BUB1 (Wolanin *et al*., 2010). Whether this reduced expression is the source for the reduced kinetochore localization of BUB1 remains to be determined (Figure 3, A and B, and Supplemental Figures S2E, and S4, A and B).

fixation. (G) Relative pixel intensity of kinetochore-localized PLK1, normalized to CENP-U WT + GFP control, from F. Each data point represents a single cell. Data points from the different experimental replicates are indicated by color. Red bars indicate mean. n = ~40 cells per group across two experimental replicates. One-way ANOVA was performed (**** = < 0.0001). Red bars indicate mean. Scale bars, 10 μM.

Understanding the basis of this perturbation, and whether this exists in other cell lines that require the CENP-O complex, will be an important topic for future work.

Core cellular processes, such as cell division, are typically considered to display similar mechanisms and requirements across cell lines. In contrast, these findings support the existence of diverse cell division behaviors and requirements across human cell lines. This work also highlights the advantage of comparing differential requirements across cell lines or cell types to determine the underlying basis for core cellular processes, including the functions of proteins that historically have been difficult to characterize. Cell line-specific susceptibilities can also identify vulnerabilities that could be exploited to screen for and develop treatment strategies for difficult to manage diseases. Such strategies could be especially beneficial in the treatment of chronic and acute myeloid leukemias, cancers that have been notoriously difficult to treat.

MATERIALS AND METHODS

Cell culture

The inducible Cas9 hTERT-RPE-1 (cTT33.1), HeLa (cTT20.11), and K-562 (cKC363) cell lines were generated by transposition as described previously (McKinley *et al.*, 2015; McKinley and Cheeseman, 2017) and are neomycin resistant. Cell lines were tested monthly for mycoplasma contamination. Inducible knockouts for CENP-O and CENP-U in HeLa (CENP-O:cKM160, CENP-U:cALN42), RPE-1 (CENP-O:cALN64, CENP-U:cALN66), and K-562 (CENP-O:cALN4, CENP-U:cALN153) cell lines were created by cloning and introducing pLenti-sqRNA (puromycin resistant; McKinley *et al.*, 2015) into the inducible Cas9 cell lines by lentiviral transduction (Wang *et al.*, 2015) using sgRNAs targeting CENP-O (CACCGTTTACGGGATCTGCTCACT) or CENP-U (CACCG AGACTTACTGATGCTCTAGG) (McKinley *et al.*, 2015). Cells were then selected with 0.35 mg/ml (HeLa), 3 mg/ml (RPE-1), or 3 mg/ml (K-562) puromycin for 14 d. The HeLa CENP-O stable knockout cell line (cKM212) was described previously (McKinley *et al.*, 2015).

Clonal cell lines expressing GFP^{LAP} fusions for BUB1, and BUB1-T609A were generated using retroviral infection in HeLa and K-562 cells as described previously (Cheeseman and Desai, 2005). The BUB1 and BUB1-T609A templates are resistant to siRNA targeted by mutation of the utilized siRNA target sequence (CTG TAC ATT GCC TGG GCG GGG to CTC TAT ATC GCT TGG GCC GGA). HeLa CENP-O knockout (cKM212) and control (cTT20.11), or K-562 CENP-U inducible knockout (cALN153) and control (cKC363) cell lines were transfected with retrovirus carrying the transgenes (pIC242: GFP, pALN24: GFP-BUB1, pALN25: GFP-BUB1-T609A) and selected with 2 mg/ml (HeLa) or 8 mg/ml (K-562) Blasticidin (Life Technologies; Cheeseman and Desai, 2005).

HeLa and RPE-1 cell lines were cultured in DMEM supplemented with 10% tetracycline-free fetal bovine serum (FBS), 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM l-glutamine (complete media) at 37°C with 5% CO_2. K-562 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% tetracycline-free FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM l-glutamine at 37°C with 5% CO_2. For knockout experiments, HeLa cells were plated on polylysine-coated coverslips, or uncoated coverslips for hTERT-RPE-1 cell lines, and 1 µg/ml doxycycline hyclate (Sigma) was added to cells at 24-h intervals for 3 d, with fixation on the fifth day. K-562 cell lines were cultured in the absence of coverslips, with doxycycline added as described above. On the fifth day, K-562 cell lines were adhered to polylysine-coated coverslips via centrifugation at 2250 rpm for 30 min at 37°C, followed by incubation at 37°C with 5% CO_2 for 1 h before fixation.

siRNAs and drug treatment

siRNAs against BUB1 (GAGUGAUCACGAUUUCUAUUU), INCENP (UGACACGGAGAUUGCCAACUU), and MAD2 (UACGGACUCACCUUGCUUGUU), and a nontargeting control were obtained from Dharmacon. RNAi experiments were conducted using Lipofectamine RNAi MAX and reduced serum OptiMEM (Life Technologies). Media was replaced with complete media 24 h after siRNA addition. Cells were assayed 48 h after transfection. To synchronize cells in mitosis, S-trityl-L-cysteine (STLC) was added to cells at 10 µM overnight.

Immunofluorescence, microscopy, and Western blotting

Cells on coverslips were fixed in 0.5% Triton X-100 + 4% formaldehyde for 10 min at room temperature. Coverslips were then washed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, then blocked with AbDil (3% bovine serum albumin [BSA], 1× Tris-buffered saline [TBS], 0.1% Triton X-100, 0.1% Na azide) for 30 min. Immunostaining was performed by incubating coverslips in primary antibody diluted in AbDil for 1 h at room temperature followed by three consecutive washes in PBS containing 0.1% Triton X-100. After washing, secondary antibodies were diluted 1:300 in AbDil and the sample was incubated for 1 h at room temperature followed by three consecutive washes in PBS containing 0.1% Triton X-100. The coverslips were next mounted in PPDM (0.5% *p*-phenylenediamine and 20 mM Tris-Cl, pH 8.8, in 90% glycerol) onto coverslips.

The following primary antibodies were used for immunofluorescence and Western blotting: PLK1 (1:200; SantaCruz; sc-17783), BUB1 (1:200; Abcam; ab54893), anti-centromere antibodies (ACA; 1:200; Antibodies; 15-234), INCENP (1:1000; Abcam; ab36453). Microtubules were stained with DM1α (1:1000 IF, 1:10,000 WB; Sigma; T6199). To increase GFP signal in select experiments, an anti-GFP V_HN nanobody conjugated to Alexa Fluor 488 was included as indicated (Chromotek; GBA-488-100). Generation of the CENP-O-P antibody was previously described and was prepared against full-length CENP-O/P-His expressed in *Escherichia coli* (McKinley *et al.*, 2015) and used at 1 µg/ml. Generation of the NDC80 "Bonsai" antibody was previously described (Schmidt *et al.*, 2012) and used at 1 µg/ml. DNA was visualized using 10 µg/ml Hoechst (Sigma-Aldrich). Cy2, Cy3-, and Cy5-conjugated secondary antibodies were obtained from Jackson Laboratories and used at 1:300. Immunofluorescence cell images were acquired on a DeltaVision Core deconvolution microscope (Applied Precision) equipped with a CoolSnap HQ2 CCD camera and deconvolved where appropriate. Approximately 35 Z-sections were acquired at 0.2-µm steps using a 100×, 1.4 numerical aperture (NA) Olympus U-PlanApo objective or a 60×, 1.42 NA Olympus U-PlanApo objective.

For Western analysis of CENP-O knockout cells, Western blotting was performed on 12% SDS–PAGE gels using 1-h semidry transfer with 3% BSA (Sigma) in TBS + 0.5% Tween-20 as a blocking agent.

Quantification and statistical analysis

Quantification of fluorescence intensity was conducted on unprocessed, maximally projected images using FIJI/image J. For image quantification, all images for comparison were acquired using the same microscope and acquisition settings. For quantification of metaphase alignment, cells were defined as misaligned if at least one off-axis chromosome was observed. Only cells with mature spindle structures were evaluated. Due to the nature of the inducible knockout system, a subset of Cas9 cleavage events will be repaired in a manner that retains the open reading frame resulting in a mixed population of cells. To ensure accurate representation of

this mixed population, the first 100 diving cells observed were analyzed from each experimental group, for each biological replicate. For analysis of BUB1, INCENP, PLK1, and NDC80 intensity at kinetochores, 10 individual kinetochores were selected at random with 4-pixel-diameter circles and the total integrated intensity was measured. Background correction was performed by selecting a nearby nonkinetochore region of equal size for each kinetochore and subtracting its integrated intensity from that of the kinetochore region. The average of all kinetochores was then determined per cell, with approximately 20–25 cells analyzed for each condition per experiment. For normalization of BUB1 and PLK1 levels against NDC80 values, the average PLK1 or BUB1 value (after background subtraction as noted above) was divided by the average NDC80 kinetochore value (with background subtraction) within the same cell. Statistical analyses were performed using Prism (GraphPad Software). Details of statistical tests and sample sizes are provided in the figure legends. An additional ANOVA statistical analysis of Figures 3, E–G, and 4, A and B is available in the included PRISM file. Data is presented as the raw kinetochore intensity measurements along with corresponding ANOVA results.

ACKNOWLEDGMENTS

We thank the members of the Cheeseman lab, as well as Karen Schindler, for their support and input. This work was supported by grants to I.M.C. from the National Institutes of Health/National Institute of General Medical Sciences (Grant no. R35GM-126930) and National Science Foundation (Grant no. 2029868), a Pilot award from the Global Consortium for Reproductive Longevity and Equity, and a Damon Runyon postdoctoral fellowship to A.L.N.

REFERENCES

Barr FA, Sillje HH, Nigg EA (2004). Polo-like kinases and the orchestration of cell division. Nat Rev Mol Cell Biol 5, 429–440.

Broad D (2020). DepMap 20Q2 Public. https://depmap.org

Cheeseman IM, Desai A (2005). A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. Sci STKE 2005, pl1.

Chen Q, Zhang M, Pan X, Zhou L, Yan H, Wang F (2021). Molecular mechanism underlying the non-essentiality of Bub1 for the fidelity of chromosome segregation in human cells. bioRxiv, 429225.

Cheeseman IM, Desai A (2008). Molecular architecture of the kinetochore-microtubule interface. Nat Rev Mol Cell Biol 9, 33–46.

Colicino EG, Hehnly H (2018). Regulating a key mitotic regulator, polo-like kinase 1 (PLK1). Cytoskeleton (Hoboken) 75, 481–494.

Combes G, Alharbi I, Braga LG, Elowe S (2017). Playing polo during mitosis: PLK1 takes the lead. Oncogene 36, 4819–4827.

Elowe S, Hummer S, Uldschmid A, Li X, Nigg EA (2007). Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. Genes Dev 21, 2205–2219.

Foltz DR, Jansen LE, Black BE, Bailey AO, Yates JR 3rd, Cleveland DW (2006). The human CENP-A centromeric nucleosome-associated complex. Nat Cell Biol 8, 458–469.

Goto H, Kiyono T, Tomono Y, Kawajiri A, Urano T, Furukawa K, Nigg EA, Inagaki M (2006). Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. Nat Cell Biol 8, 180–187.

Hara M, Fukagawa T (2017). Critical Foundation of the kinetochore: the constitutive centromere-associated network (CCAN). Prog Mol Subcell Biol 56, 29–57.

Hori T, Okada M, Maenaka K, Fukagawa T (2008). CENP-O class proteins form a stable complex and are required for proper kinetochore function. Mol Biol Cell 19, 843–854.

Kang YH, Park CH, Kim TS, Soung NK, Bang JK, Kim BY, Park JE, Lee KS (2011). Mammalian polo-like kinase 1-dependent regulation of the PBIP1-CENP-Q complex at kinetochores. J Biol Chem 286, 19744–19757.

Kang YH, Park JE, Yu LR, Soung NK, Yun SM, Bang JK, Seong YS, Yu H, Garfield S, Veenstra TD, Lee KS (2006). Self-regulated Plk1 recruitment to kinetochores by the Plk1-PBIP1 interaction is critical for proper chromosome segregation. Mol Cell 24, 409–422.

Lee KS, Park JE, Kang YH, Zimmerman W, Soung NK, Seong YS, Kwak SJ, Erikson RL (2008). Mechanisms of mammalian polo-like kinase 1 (Plk1) localization: self- versus non-self-priming. Cell Cycle 7, 141–145.

Lera RF, Burkard ME (2012). High mitotic activity of Polo-like kinase 1 is required for chromosome segregation and genomic integrity in human epithelial cells. J Biol Chem 287, 42812–42825.

Lera RF, Norman RX, Dumont M, Dennee A, Martin-Koob J, Fachinetti D, Burkard ME (2019). Plk1 protects kinetochore-centromere architecture against microtubule pulling forces. EMBO Rep 20, e48711.

Lera RF, Potts GK, Suzuki A, Johnson JM, Salmon ED, Coon JJ, Burkard ME (2016). Decoding Polo-like kinase 1 signaling along the kinetochore-centromere axis. Nat Chem Biol 12, 411–418.

Marchetti F, Venkatachalam S (2010). The multiple roles of Bub1 in chromosome segregation during mitosis and meiosis. Cell Cycle 9, 58–63.

McKinley KL, Cheeseman IM (2017). Large-scale analysis of CRISPR/Cas9 cell-cycle knockouts reveals the diversity of p53-dependent responses to cell-cycle defects. Dev Cell 40, 405–420.e402.

McKinley KL, Sekulic N, Guo LY, Tsinman T, Black BE, Cheeseman IM (2015). The CENP-L-N complex forms a critical node in an integrated meshwork of interactions at the centromere-kinetochore interface. Mol Cell 60, 886–898.

Meyers RM, Bryan JG, McFarland JM, Weir BA, Sizemore AE, Xu H, Dharia NV, Montgomery PG, Cowley GS, Pantel S, *et al*. (2017). Computational correction of copy number effect improves specificity of CRISPR-Cas9 essentiality screens in cancer cells. Nat Genet 49, 1779–1784.

Minoshima Y, Hori T, Okada M, Kimura H, Haraguchi T, Hiraoka Y, Bao YC, Kawashima T, Kitamura T, Fukagawa T (2005). The constitutive centromere component CENP-50 is required for recovery from spindle damage. Mol Cell Biol 25, 10315–10328.

Park CH, Park JE, Kim TS, Kang YH, Soung NK, Zhou M, Kim NH, Bang JK, Lee KS (2015). Mammalian Polo-like kinase 1 (Plk1) promotes proper chromosome segregation by phosphorylating and delocalizing the PBIP1. CENP-Q complex from kinetochores. J Biol Chem 290, 8569–8581.

Pesenti ME, Prumbaum D, Auckland P, Smith CM, Faesen AC, Petrovic A, Erent M, Maffini S, Pentakota S, Weir JR, *et al*. (2018). Reconstitution of a 26-subunit human kinetochore reveals cooperative microtubule binding by CENP-OPQUR and NDC80. Mol Cell 71, 923–939.e910.

Qi W, Tang Z, Yu H (2006). Phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 is required for the kinetochore localization of Plk1. Mol Biol Cell 17, 3705–3716.

Schmidt JC, Arthanari H, Boeszoermenyi A, Dashkevich NM, Wilson-Kubalek EM, Monnier N, Markus M, Oberer M, Milligan RA, Bathe M, *et al*. (2012). The kinetochore-bound Ska1 complex tracks depolymerizing microtubules and binds to curved protofilaments. Dev Cell 23, 968–980.

Singh P, Pesenti ME, Maffini S, Carmignani S, Hedtfeld M, Petrovic A, Srinivasamani A, Bange T, Musacchio A (2020). BUB1 and CENP-U, primed by CDK1, are the main PLK1 kinetochore receptors in mitosis. Mol Cell 81, 67–87.e9.

Wang T, Birsoy K, Hughes NW, Krupczak KM, Post Y, Wei JJ, Lander ES, Sabatini DM (2015). Identification and characterization of essential genes in the human genome. Science 350, 1096–1101.

Wolanin K, Magalska A, Kusio-Kobialka M, Podszywalow-Bartnicka P, Vejda S, McKenna SL, Mosieniak G, Sikora E, Piwocka K (2010). Expression of oncogenic kinase Bcr-Abl impairs mitotic checkpoint and promotes aberrant divisions and resistance to microtubule-targeting agents. Mol Cancer Ther 9, 1328–1338.