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Cell cycle-coupled CK1δ turnover, autoinhibition, and activity

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eLife Assessment

This manuscript presents **valuable** experimental results describing the localisation and regulation of casein kinase CK1δ during the cell cycle. During the G2 phase of the cell cycle, the autophosphorylation of the C-terminal tail stabilises and inhibits the kinase which might protect CK1δ in the subsequent G1 phase. The results may be of interest to researchers working on casein kinase function and regulation but due to lack of some controls remain **incomplete**.

[Editors' note: this paper was reviewed by *Review Commons*.]

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Abstract

Casein kinase 1δ (CK1δ) is a ubiquitously expressed kinase involved in diverse cellular processes, including cell cycle regulation. CK1δ activity is attenuated by (auto)phosphorylation. However, inhibitory phosphorylation is efficiently opposed by cellular phosphatases as CK1δ accumulates in its hypophosphorylated, active state. CK1δ is a target of the nuclear ubiquitin ligase APC/C-CDH1, yet the kinase is apparently stable. Thus, the physiological relevance of CK1δ (auto)phosphorylation, autoinhibition, and regulated turnover has remained unclear. Here we show that CK1δ activity and abundance are coordinated in a cell cycle-dependent manner. During G1, assembled CK1δ kinase is stable while free active kinase is degraded. In S phase, unassembled CK1δ seems to be no longer degraded, likely to support functions in DNA damage signaling. Upon mitotic entry, the downregulation of phosphatases promotes CK1δ (auto)phosphorylation and consequent autoinhibition, thereby preserving a pool of kinase to rapidly reestablish the post-mitotic steady state.

Introduction

Casein kinase 1 (CK1) is an evolutionarily conserved serine/threonine kinase found throughout the eukaryotic lineage[1], with homologs in yeast[2], fungi[3], plants[4], and animals[5]. CK1 isoforms act as effectors in a variety of cellular signalling pathways[6,7]. All members of the CK1 family share a highly conserved, structured catalytic domain and a divergent, largely disordered C-terminal tail[8,9]. CK1 enzymes are phosphate-directed kinases displaying high affinity for motifs pre-phosphorylated (primed) at the -3 and to a lesser extent also the -4 position with the consensus pS/T-X-X-(X)-S/T but display much lower activity towards unprimed sites[10–12]. This specificity is conferred by a dedicated phosphate-binding pocket that correctly orients the primed substrate[11]. CK1s are considered constitutively active because they do not have regulatory subunits and are not activated by phosphorylation of their activation loop[13].

Among the seven CK1 isoforms in humans (α, β, γ1, γ2, γ3, δ, and ε), CK1δ and CK1ε play crucial roles in regulating the circadian clock[5,10,12], cytoskeletal organization[14,15], cell cycle[16,17], and development[18–20]. CK1δ and CK1ε activity is attenuated by phosphorylation of their C-terminal tail[8,9]. Autoinhibition occurs via a competitive mechanism in which the phosphorylated tail blocks the active site[21,22]. Inhibitory tail phosphorylation is mediated by autophosphorylation and phosphorylation by other cellular kinases[7,12]. Although C-terminal tail phosphorylation inhibits

CK1δ/ε in vitro, the kinases undergoes futile cycles of phosphorylation and dephosphorylation[23], keeping the kinase in a dephosphorylated, active state[24]. CK1δ/ε activity is controlled by several mechanisms, including sequestration[7], leaving the physiological significance of this inhibitory mechanism unclear.

CK1δ is shuttling between nucleus and cytoplasm and localizes dynamically to the centrosome and pericentrosomal region[15,25,26]. Catalytically inactive CK1δ mutants accumulate in the nucleus and nuclear export of CK1δ depends on kinase activity[27,28]. CK1δ is a target of the nuclear ubiquitin ligase anaphase-promoting complex/cyclosome in association with its coactivator CDH1 (APC/C-CDH1)[29], which is active from late anaphase/telophase through G1 and inactivated when the cell cycle progresses into S phase[30]. Yet, CK1δ appears to be stable[31,32], raising the question about the physiological conditions under which APC/C-CDH1 targets the kinase.

We have recently analyzed the posttranslational regulation of CK1δ in unsynchronized cells, which are mainly in the G1 phase of the cell cycle[28]. These findings indicate that CK1δ is stabilized through assembly with binding partners such as the circadian clock protein PER2. In contrast, unassembled CK1δ, which accumulates at detectable levels upon overexpression, is rapidly degraded. This suggests that APC/C-CDH1 selectively targets the unassembled kinase, thereby limiting aberrant CK1δ activity. Here, we expand our analysis of CK1δ regulation in G1 and investigate how CK1δ stability and localization are controlled across the cell cycle.

Results

Subcellular distribution of CK1δ is dynamic

We have recently shown that CK1δ expressed at physiological levels is stable while overexpressed CK1δ which is not assembled with binding partners such as PERIOD2, is rapidly degraded, preferentially in the nucleus in a manner dependent on its kinase is activity[28]. To characterize in more detail the subcellular dynamics, synthesis and turnover of endogenous CK1δ we performed immunofluorescence (IF) of U2OStx cells. In untreated cells, CK1δ was highly concentrated in the pericentrosomal region (Figs. 1A, C), consistent with its reported localization at the centrosome and Golgi apparatus[7,33], and was also detectable in the nucleus, consistent with its reported association with PERIOD proteins[32].

We then asked whether the subcellular localization of CK1δ is static or dynamic. It has been shown that catalytically inactive CK1δ mutants accumulate in the nucleus[27] and we have previously shown that kinase inhibition with PF670462, a specific CK1δ/ε inhibitor[34], inhibits nuclear export of CK1δ and, in addition, protects the kinase from degradation. Therefore, to trap and stabilize shuttling CK1δ in the nucleus, U2OStx cells were treated for 4 h with PF670462 together with cycloheximide (CHX) to distinguish relocalization from de novo protein synthesis. Under these conditions, the localization of CK1δ at the pericentrosomal region was reduced and the kinase accumulated in the nucleus (Figs. 1A, C). These data demonstrate that the pericentrosomal localization of CK1δ is dynamic and in equilibrium with the nuclear pool. A fraction of CK1δ remained associated with the pericentrosomal region after the 4 h treatment with PF670462 (Fig.1 C).

Next, we analyzed U2OStx cells stably overexpressing doxycycline (DOX)-inducible FLAG-tagged CK1δ. Expression of CK1δ-FLAG was induced for 24 h with DOX to generate a pool of unassembled kinase and analysed by IF. Despite overexpression, the subcellular distribution of CK1δ-FLAG was similar to that of CK1δ in control cells, displaying pronounced concentration of CK1δ-FLAG in the pericentrosomal region (Fig. 1B). Treatment with PF670462 resulted in nuclear accumulation of CK1δ-FLAG and reduction of pericentrosomal staining (Figs. 1B, D). These data indicate that pericentrosomal and nuclear CK1δ exist in a dynamic equilibrium, regardless of whether the kinase is expressed at physiological levels or overexpressed.

Thus, at steady state, the subcellular levels of endogenous or overexpressed CK1δ are not static but are dynamically maintained by the balance between subcellular shuttling, binding equilibria with the pericentrosomal region. We have recently shown that unassembled CK1δ is rapidly degraded

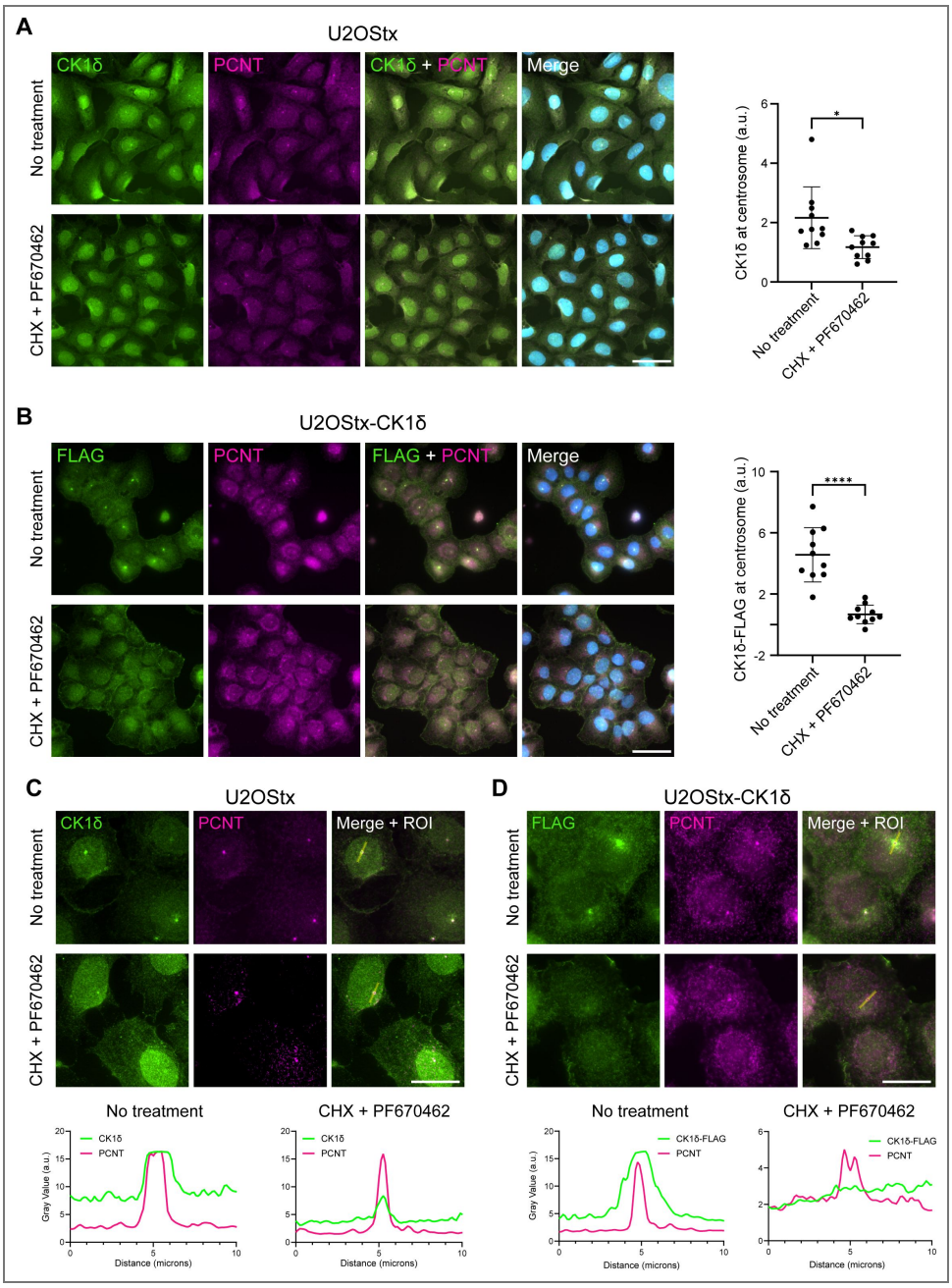

Figure 1. CK1δ inhibition results in decreased centrosomal localization.

(A) U2OStx cells were treated with a combination of CHX and PF670462 for 4 h to inhibit protein synthesis and kinase activity or left untreated. Endogenous CK1δ and pericentrin (PCNT) were detected by IF. Green (AF488): anti-CK1δ, magenta (Cy3): anti-PCNT. Nuclei were identified with DAPI. In untreated cells, CK1δ exhibited clear nuclear and pericentrosomal staining. In CHX + PF670462-treated cells, pericentrosomal localization of CK1δ was decreased and CK1δ accumulated in the nucleus. Scale bar = 50 μm. Right panel: Density above surrounding background of CK1δ at pericentrosomal region. Quantification of n = 10 cells, Welch's t-test. * indicates p-value < 0.05. (B) DOX-induced U2OStx_CK1δ cells were either left untreated or treated with CHX and PF670462 for 4 h and analyzed as described above. Green (AF488): CK1δ-FLAG was detected with anti-FLAG antibody. Magenta (CY3): anti-PCNT. In untreated cells, CK1δ-FLAG was concentrated at the pericentrosomal region. In CHX + PF670462-treated cells, pericentrosomal localization of CK1δ-FLAG was decreased and CK1δ-FLAG accumulated in the nucleus. Right panel: Density above surrounding background of CK1δ-FLAG at pericentrosomal region. Quantification of n = 10 cells. Welch's t-test. **** indicates p-value < 0.0001. U2OStx cells (C) and DOX-induced U2OStx_CK1δ cells (D) were analyzed as described in (A) and (B), respectively. Fiji-based multi-channel line plot analysis showing the fluorescence intensity profiles of CK1δ (C) and CK1δ-FLAG (D) relative to PCNT along the indicated region-of-interest (ROI) in representative untreated cells and cells treated with CHX or PF670462. Scale bar = 20 μm.

in the nucleus[28]. Our data suggest that under physiological expression levels, rebinding of CK1δ to the pericentrosomal region constitutes the dominant pathway, effectively outcompeting nuclear degradation, while overexpression promotes degradation of free nuclear CK1δ. Such a mechanism would enable continuous monitoring and regulation of CK1δ levels while maintaining a low abundance of unassembled, potentially harmful kinase. Because free CK1δ levels are kept low and pericentrosomal binding sites are not saturated[28], the pericentrosomal CK1δ pool may function as a buffered reservoir that can supply newly emerging binding partners such as PERIOD proteins, when they accumulate at higher levels than free CK1δ.

To test this hypothesis, we employed U2OStx cells stably expressing mKate2-tagged Cryptochrome 1 (mK2-CRY1). These U2OStx-mK2-CRY1 cells were transfected with PER2 to rapidly overexpress a binding partner of CK1δ. As recently shown[28], overexpressed PER2 and mK2-CRY1 mutually stabilize each other and co-accumulate in nuclear foci, whereas in untransfected cells mK2-CRY1 alone is unstable, expressed at a low level and homogeneously distributed throughout the nucleus (Fig. 2A). Thus, the formation of nuclear mK2-CRY1 foci serves as an indicator of PER2 transfection[28]. Because U2OStx cells exhibit low transfection efficiency, only a fraction of U2OS-mK2-CRY1 cells expressed PER2. This allowed a side-by-side comparison between endogenous CK1δ in PER2-expressing cells identified by nuclear mK2-CRY1 foci from non-expressing cells within the same population. In untransfected cells, endogenous CK1δ was concentrated at the single centrosome (Fig. 2B, red arrows). In contrast, in PER2-transfected cells, CK1δ accumulated at elevated levels and colocalized with mK2-CRY1 in PER2-dependent nuclear foci (Fig. 2B). Notably, all nuclear CK1δ foci were mK2-CRY1 positive, whereas CK1δ foci lacking mK2-CRY1, which would indicate pericentrosomal localization of CK1δ, were not detected in PER2-transfected cells. This change in CK1δ localization upon formation of nuclear mK2-CRY1 foci is consistent and can be seen in multiple fields-of-view (FOV). Together, these findings suggest that PER2 overexpression competes for CK1δ binding and, when expressed at very high levels, can even displace CK1δ from the pericentrosomal region.

Dephosphorylation of CK1δ by cellular phosphatases

Phosphorylation of CK1δ at the kinase C-terminal tail can be stabilized and detected by treating cells with phosphatase inhibitors[35]. We compared the kinetics of tail phosphorylation upon treatment of cells with Calyculin A (CalA), a potent inhibitor of the protein phosphatases PP1 and PP2A, and okadaic acid (OA), which inhibits PP2A more effectively than PP1[36]. For this purpose, we used U2OStx stably expressing under a doxycycline (DOX)-inducible promoter either FLAG-tagged wild-type CK1δ or its catalytically inactive (kinase-dead) version, CK1δ-K38R[12]. In U2OStx_CK1δ cells, both CalA and OA inhibited CK1δ dephosphorylation and led to the accumulation of elevated levels of hyperphosphorylated CK1δ (Fig. 3A), suggesting that phosphorylation protects the overexpressed kinase from degradation. CalA does not inhibit PP2C or protein tyrosine phosphatases and is largely ineffective against PP2B and PP7. Thus, the sensitivity of CK1δ dephosphorylation to CalA suggests PP1, PP2A, PP4, and PP5 as likely candidates contributing to dephosphorylation[36]. Although sensitivity to CalA and OA cannot rigorously distinguish among these phosphatases, the high OA concentrations required to inhibit CK1δ dephosphorylation suggest the involvement of PP1, which is 10 - 100-fold less sensitive to OA than PP2A, PP4, or PP5[35,36]. Thus, CK1δ is actively maintained in a dephosphorylated state, most likely through the contribution of PP1, with possible additional contributions from PP2A, PP4, and PP5. PP1 has previously suggested as a major phosphatase of CK1δ/ε[37]. Notably, PP1, PP2A, and PP4 activities are downregulated during mitosis[38,39], pointing to a potential physiological role for CK1δ tail phosphorylation and autoinhibition during this stage of the cell cycle.

To analyze whether the electrophoretic mobility shift induced by CalA treatment was indeed caused by phosphorylation of the CK1δ tail, we generated a U2OStx cell line expressing a CK1δ variant in which all serine and threonine residues in the C-terminal tail were replaced by alanine ($CK1\delta_A$). Treatment of these cells with CalA did not induce an electrophoretic mobility shift of the tail mutant kinase (Fig. 3B).

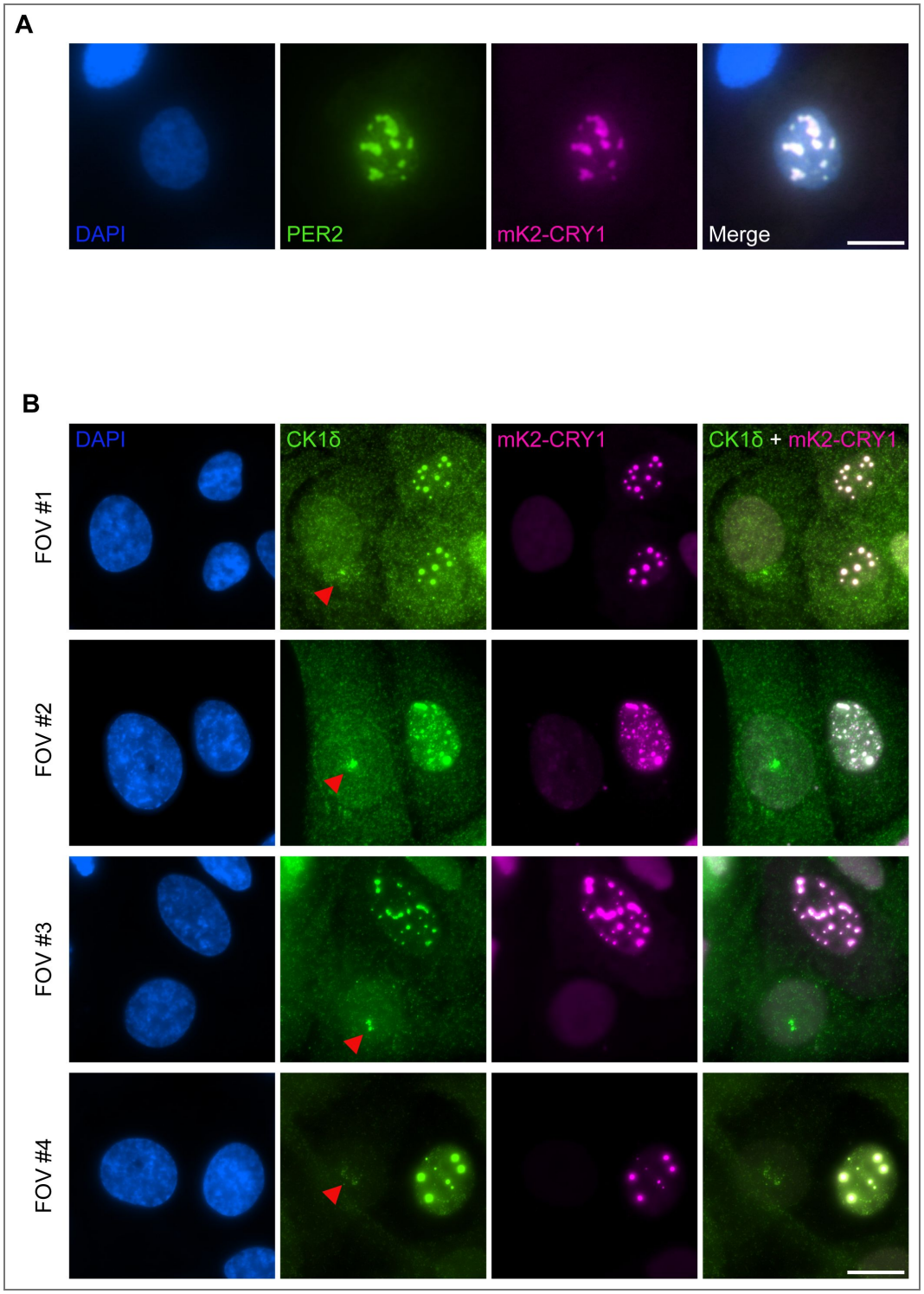

Figure 2. PER2 overexpression displaces CK1δ from the centrosome

U2OStx_mK2-CRY1 cells were transfected with PER2, followed by DOX induction. (A) IF for PER2 shows the formation of nuclear foci containing both overexpressed PER2 and mK2-CRY1 as reported previously[28]. Scale bar = 20 μm. (B) IF for CK1δ shows that the kinase expressed from its endogenous locus is recruited to the nuclear PER2-CRY1 foci and depleted from the pericentrosomal region, in agreement with previous observations[28]. Four FOVs are shown. Presence and absence of nuclear mK2-CRY1 foci indicate PER2-transfected and untransfected cells, respectively. Pericentrosomal CK1δ localization, accessed by CK1δ positive (green) and CRY1 negative (magenta) foci, was observed in all untransfected cells examined (50 out of 50 cells evaluated). In PER2 and CRY1-expressing cells CK1δ accumulated in CRY1-positive (magenta) nuclear foci. No cell (0 out of 15 cells evaluated) displayed pericentrosomal localization of CK1δ (CRY1-negative focus). Fisher's exact test, $p = 4.8 \times 10^{-15}$. Scale bar = 20 μm.

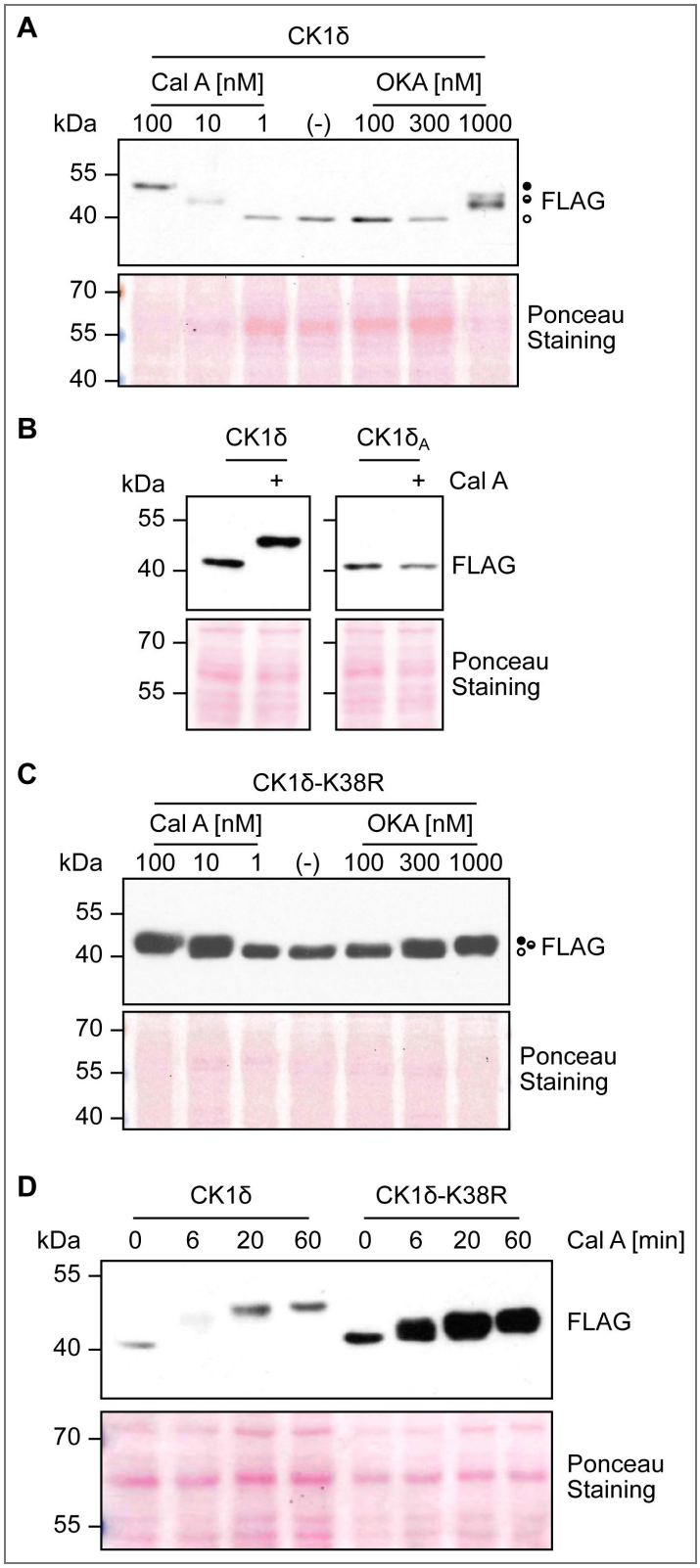

Figure 3. Phosphatase inhibition results in CK1δ phosphorylation

(A) Immunoblot analysis of CalA- and OA-induced phosphorylation of CK1δ-FLAG. Phosphorylation of CK1δ is strongly induced by CalA and, albeit with markedly lower efficiency, also by OA. n = 3. (B) Immunoblot analysis demonstrating the absence of a CalA-induced electrophoretic mobility shift in the $CK1\delta_A$ mutant kinase. n = 3. (C) Immunoblot analysis of CalA- and OA-induced phosphorylation of kinase-dead CK1δ-K38R. n = 3. (D) Time-course of CalA-induced phosphorylation of CK1δ and CK1δ-K38R. n = 3. Ponceau S staining of membranes are shown as loading control.

To separate autophosphorylation from phosphorylation by other kinases, we examined U2OStx_CK1δ-K38R cells. Upon CalA or OA treatment, the kinase-dead CK1δ-K38R accumulated at elevated levels and was also phosphorylated but to a lesser extent than wild-type CK1δ (Fig. 3C), suggesting that fewer phosphorylation sites or targeted by other kinases. Because CK1δ-K38R is already rather stable[40], the data suggest that partial tail phosphorylation is sufficient to further stabilize CK1δ-K38R. Time-course experiments revealed that CK1δ became hyperphosphorylated within 1 h of CalA treatment, with intermediate phosphorylation states visible at 6 and 20 min (Fig. 3D), in agreement with previous observations[8]. CalA-induced phosphorylation of CK1δ-K38R followed similar kinetics (Fig. 3D). We noted that treatment with a PPase inhibitor led to increased levels of overexpressed, and thus unassembled, CK1δ (Fig. 3A, D) as well as CK1δ-K38R (Fig. 3C, D), suggesting that full or partial phosphorylation of the CK1δ tail stabilizes both active and inactive forms of the kinase.

Tail phosphorylation protects CK1δ/ε from degradation

To examine in more detail how tail phosphorylation affects the turnover of unassembled CK1δ, U2OStx cells stably expressing CK1δ-FLAG were either left untreated or pre-treated for 1 h with the phosphatase inhibitor CalA, alone or together with PF670, followed by a 90-min cycloheximide (CHX) chase. In CHX-treated cells, overexpressed CK1δ was rapidly degraded, with a half-life of approximately 15 min (Fig. 4A), consistent with our previous findings[40]. The 1 h pre-treatment with CalA induced hyperphosphorylation of CK1δ, whereas co-treatment with PF670462 and CalA produced an intermediate phosphorylation state (Fig. 4A), similar to the CalA-induced phosphorylation of CK1δ-K38R, indicating that PF670462-insensitive kinases targeted a fraction of the phosphorylation sites in CK1δ tail. Both 1 h pre-treatments resulted in elevated CK1δ expression levels, indicating that most of the newly synthesized overexpressed kinase was degraded in untreated cells. This conclusion was confirmed by the CHX chase. Hyperphosphorylated CK1δ remained stable throughout the CHX chase (Fig. 4A, C), indicating that tail phosphorylation protects the overexpressed kinase from rapid degradation. CK1δ shares a high degree of sequence identity and potential functional redundancy with CK1ε, with both kinases implicated in circadian clock regulation[5,41] and Wnt signalling[42]. Treatment of cells overexpressing FLAG-tagged CK1ε with CHX, CalA, and PF670462 revealed that CK1ε turnover is also regulated in a similar manner to CK1δ (Fig. 4B, D).

CK1δ is a target of the nuclear ubiquitin ligase APC/C-CDH1[29]. To directly analyze the subcellular turnover of CK1δ, we employed U2OStx cell lines expressing mNeonGreen-tagged CK1δ fused either to an SV40 NLS or to an HIV-1 NES, which direct the kinase predominantly to the nucleus or cytosol, respectively[40]. Western blot analysis of cycloheximide-treated cell lines revealed that NLS-mNG-CK1δ was degraded more rapidly than NES-mNG-CK1δ.

Cell cycle-dependence of CK1δ expression and phosphorylation

Because CK1δ has various functions at different stages of the cell cycle, including S phase and mitosis[6,17], we investigated whether expression and phosphorylation state of CK1δ are regulated in a cell cycle-dependent manner. CK1δ is a target of the nuclear ubiquitin ligase APC/C-CDH1[29], which is active from late anaphase/telophase all the way through G1 and inactive in S phase, G2 and mitosis[30]. To analyze whether inactivation of APC/C-CDH1 affects expression levels of unassembled CK1δ, U2OStx_CK1δ were arrested at G1/S with a double thymidine block. The block was then relieved and CK1δ expression was analyzed (Fig. 5A). CK1δ levels increased after release from the G1/S arrest, compared to non-arrested cells (which are predominantly in G1) and to cells arrested at the G1/S transition (Fig. 5B). The accumulated CK1δ remained dephosphorylated, indicating that the phosphatases acting on the kinase tail were active during S phase. These findings suggest that overexpressed, and thus unassembled CK1δ accumulates in a dephosphorylated, active state and escapes nuclear degradation at cell cycle states when APC/C-CDH1 is inactive. The data suggest that inactivation of APC/C-CDH1 in S-phase may protect free nuclear CK1δ to facilitate its functions in DNA damage repair and checkpoint signalling.

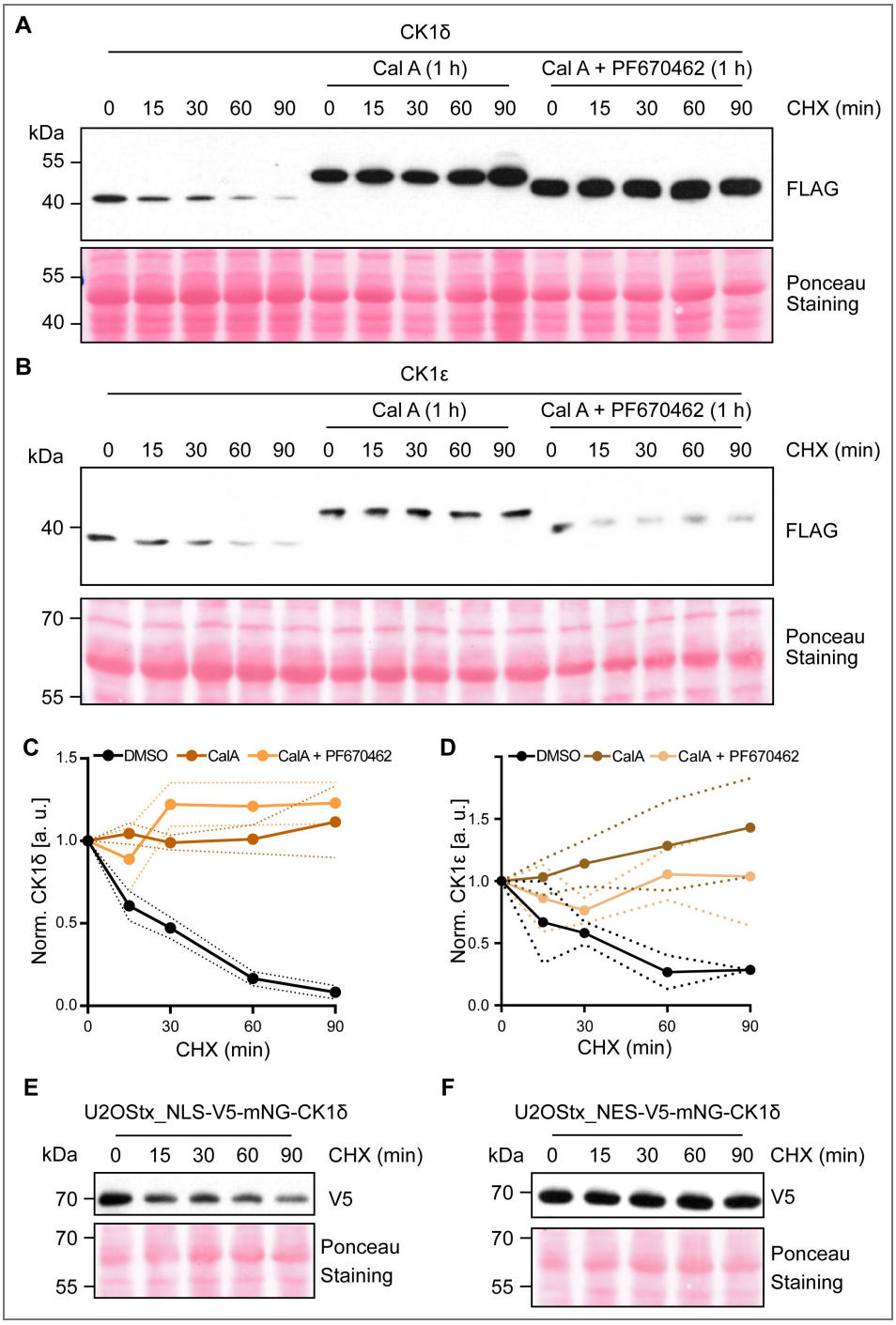

Figure 4. CK1δ/ε phosphorylation stabilizes overexpressed kinase.

(A) Phosphorylation protects overexpressed CK1δ from degradation. U2OStx_CK1δ cells were incubated with cycloheximide (CHX) (left part) or pretreated for 1 h with CalA (middle part) or with CalA + PF670462 (right part). CK1δ was analyzed at the indicated time points after CHX addition. Overexpressed CK1δ is rapidly degraded. CalA and CalA + PF670462 induce maximal and intermediate phosphorylated CK1δ and stabilize the kinase. n = 3. (B) CalA and CalA + PF670462 induce phosphorylation of CK1ε and stabilize the kinase. n = 3. (C) Densitometric quantification of n=3 Western blots (see A) shown as mean ± SD. Overexpressed unphosphorylated CK1δ is degraded with a half-life of about 15 min. Both CalA and CalA + PF670462 treatments stabilize the kinase. (D) Densitometric quantification of n=3 Western blots (see B) shown as mean ± SD. (E) CK1δ is preferentially degraded in the nucleus. U2OStx cells stably expressing NLS-mNG-CK1δ and NES-mNG-CK1δ were incubated with CHX. Samples were analyzed at the indicated time points after CHX addition. Overexpressed NLS-mNG-CK1δ is unstable while NES-mNG-CK1δ is stable. Ponceau S staining of membranes are shown as loading control.

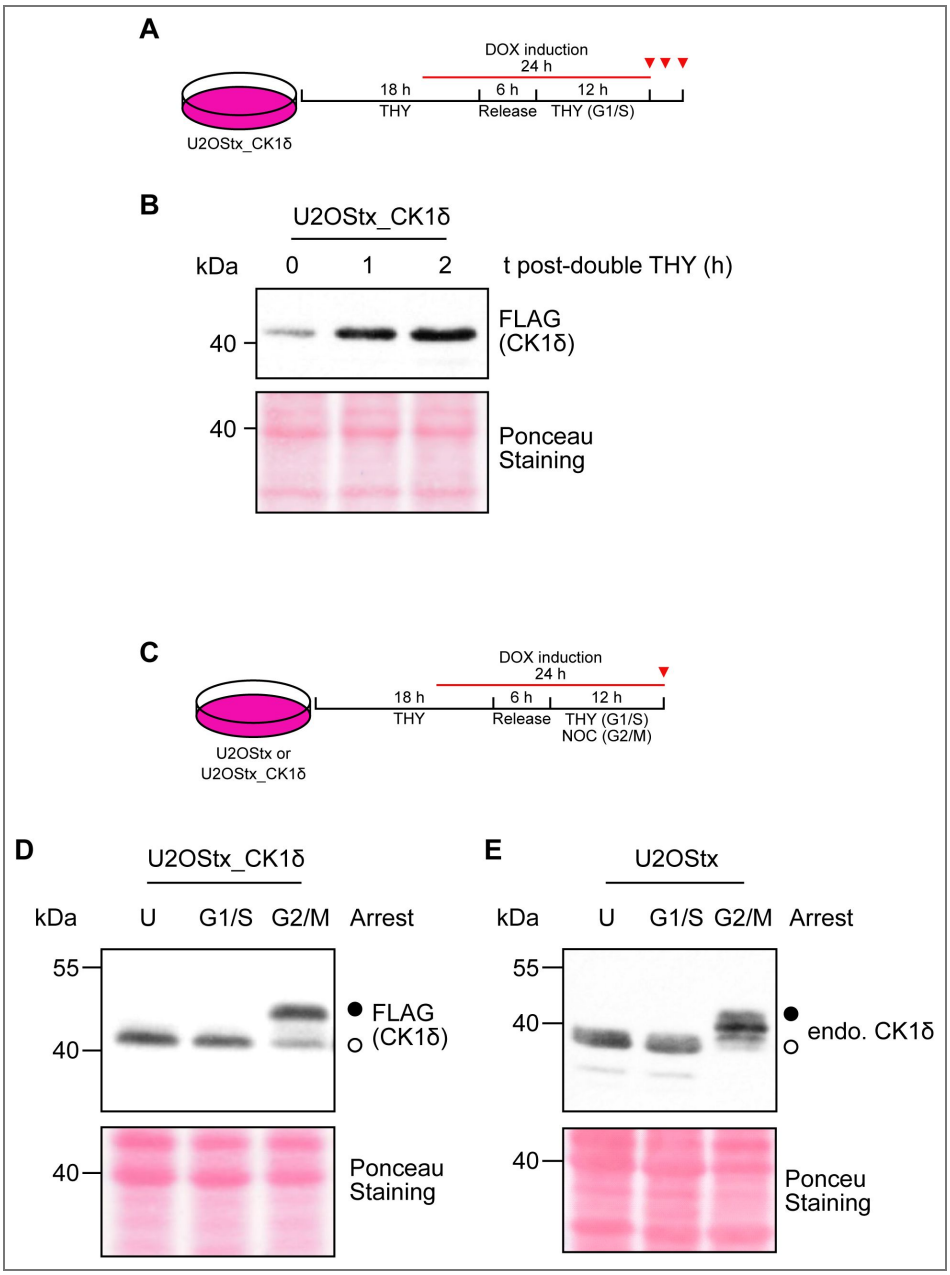

Figure 5. CK1δ is stabilized post-S phase and phosphorylated during G2/M

(A) Schematic of S phase arrest and release of U2OStx_CK1δ cells. (B) Overexpressed CK1δ accumulates post-S phase release. n = 3. (C)) Schematic of G1/S and G2/M phase arrest of U2OStx_CK1δ or U2OStx cells (D, E) Overexpressed CK1δ in U2OStx_CK1δ cells (D) and endogenous CK1δ in U2OStx cells (E) are unphosphorylated in unsynchronized and G1/S-arrested cells. G2/M-arrested cells accumulate hyperphosphorylated CK1δ. n = 3. Ponceau S staining of membranes are shown as loading control.

Upon entry into mitosis, the activity of major cellular phosphatases such as PP1 and PP2A is globally attenuated[38,39], while distinct pools of these phosphatases remain active at defined subcellular structures[43]. We reasoned that this inhibition might facilitate the accumulation of phosphorylated CK1δ. To test this, we arrested U2OStx_CK1δ cells at the G1/S boundary using a double thymidine block, and at G2/M using a thymidine-nocodazole block followed by mitotic shake-off to enrich for cells in mitosis[44,45] (Fig. 5C). CK1δ-FLAG was then analyzed via immunoblotting. In unsynchronized (U) and G1/S-arrested cells, CK1δ was hypo- or unphosphorylated. In contrast, G2/M-arrested cells accumulated tail-phosphorylated CK1δ (Fig. 5D). Similarly, endogenous CK1δ was hyperphosphorylated upon G2/M arrest of U2OStx control cells while it remained hypophosphorylated in unsynchronized and G1/S-arrested cells (Fig. 5E).

To assess the localization of CK1δ during mitosis, we performed IF on U2OStx_CK1δ cells staining for the overexpressed kinase. Based on the DAPI staining we assigned the fixed cells to distinct cell-cycle stages. In G2/prophase, identified by duplicated centrosomes and increased DNA content, CK1δ localized predominantly to structures which correspond to the duplicated centrosomes (Fig. 6A, 1st column), consistent with previous reports[15,25]. In metaphase and anaphase (Fig. 6A, 2nd column), characterized by DNA condensation, overexpressed CK1δ localized to centrosomes and polar microtubules, consistent with previous reports on the localization of endogenous kinase[14]. In addition, a considerable fraction of the overexpressed CK1δ was evenly distributed throughout the cell, suggesting that unassembled CK1δ was not targeted for degradation. During telophase and cytokinesis, as chromosome segregation and cell division progressed, CK1δ was diffusely distributed across the dividing cell (Fig. 6A, 3rd column). Although it is not possible to identify cells that have only recently exited mitosis and entered G1 in fixed samples, all G1 cells displayed a concentration of CK1δ at the pericentrosomal region (Fig. 6A, 4th column). Similar results were observed when using U2OStx cells and staining for endogenous CK1δ (Fig. 6B).

Discussion

A central and long-standing question in the regulation of casein kinase 1δ (CK1δ) concerns the physiological role of its autoinhibitory C-terminal tail phosphorylation. Biochemical studies have established that autophosphorylation of the CK1δ tail suppresses catalytic activity, suggesting an intrinsic mechanism for kinase regulation. However, this model appears difficult to reconcile with observations in cells, where CK1δ is found predominantly in a dephosphorylated, and therefore catalytically active state. This discrepancy raises a general conundrum: if CK1δ is constitutively active in vivo, how is its activity effectively regulated, and under what physiological circumstances does tail-mediated autoinhibition become relevant?

A related paradox has emerged in the circadian clock field. CK1δ plays a central role in regulating the stability and nuclear function of circadian PER proteins and is therefore expected to be readily available to ensure robust circadian timekeeping. In principle, CK1δ should not be limiting for PER phosphorylation. Yet, overexpression of CK1δ consistently accelerates the circadian clock, implying that kinase availability can influence clock speed. This finding suggests that CK1δ activity may be regulated not only by catalytic mechanisms but also by its spatial availability within the cell.

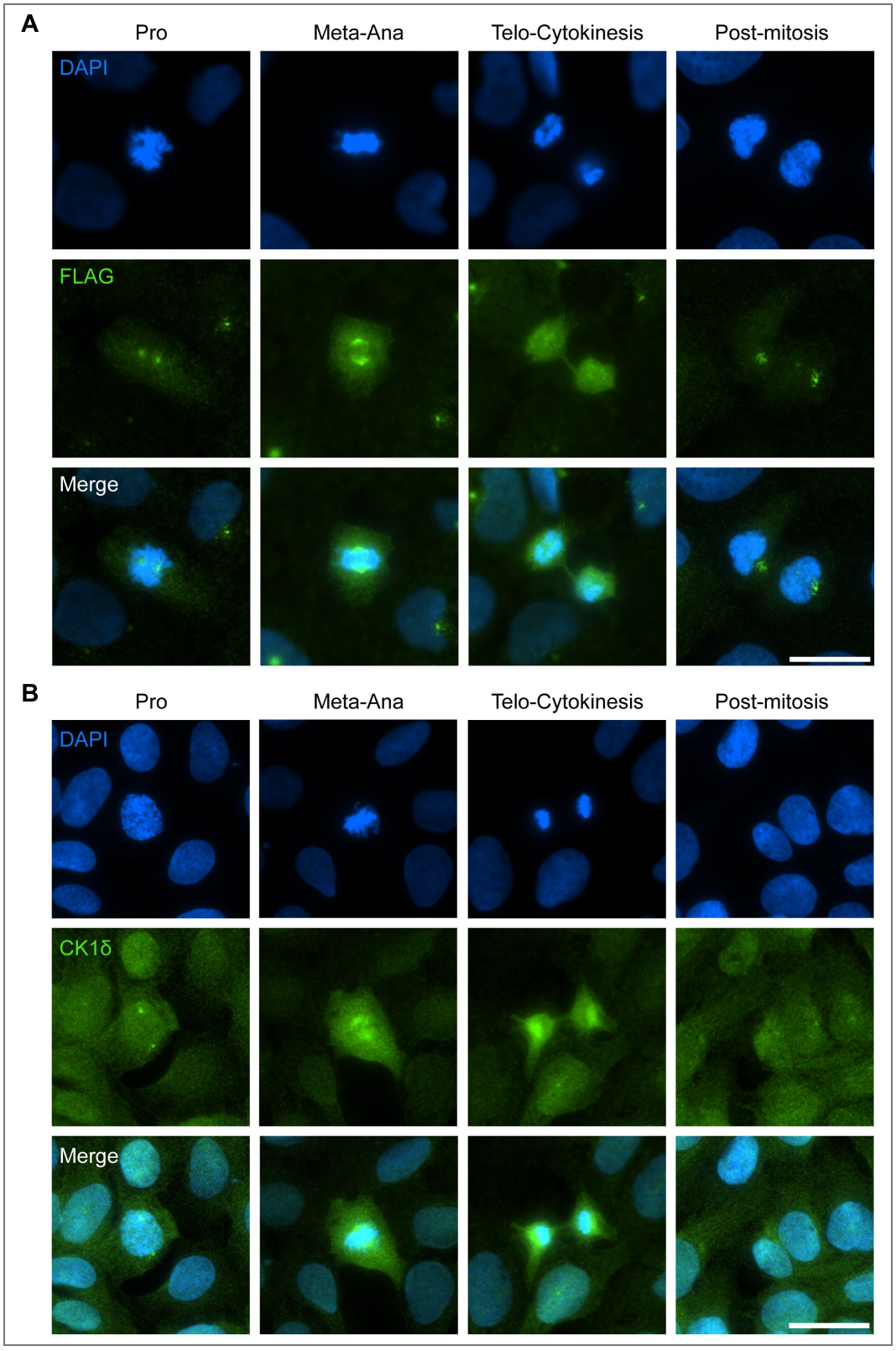

Figure 6. CK1δ localization during mitosis

(A) Upper panels: DAPI-staining of U2OStx_CK1δ cells was used to identify stages of the cell cycle. Middle panels. IF analysis of overexpressed CK1δ with FLAG antibody. Lower panels: Merged DAPI and FLAG image. Scale bar = 20 μm. (B) Upper panels: DAPI-staining of U2OStx cells was used to identify stages of the cell cycle. Middle panels. IF analysis of endogenous CK1δ with anti-CK1δ antibody. Lower panels: Merged DAPI and CK1δ image. Scale bar = 20 μm. Pro: Prophase, Meta-Ana: Metaphase-Anaphase, Telo-Cytokinesis: Telophase-Cytokinesis.

We have recently begun to address this paradox by demonstrating the importance of subcellular localization as it relates to CK1δ function and activity. We have shown that CK1δ is not limiting in the cytosol but becomes limiting in the nucleus in the context of the circadian clock[28]. This present study provides a potential explanation for how cells reconcile a low synthesis rate of CK1δ and low levels of free kinase with the need for efficient association with binding partners such as PER proteins. Our data indicate that CK1δ is dynamically stored at the pericentrosomal region, potentially forming a buffering pool that can be readily mobilized to bind to its partners. Such a mechanism would allow the cell to maintain low levels of free, potentially harmful, active kinase while simultaneously ensuring that CK1δ is readily available to newly synthesized PER proteins. In this way, pericentrosomal sequestration could function as a buffering system that couples limited CK1δ production to the rhythmically high demand for PER binding and phosphorylation in circadian timekeeping.

Here, we have also addressed the fate of CK1δ across the cell cycle. Because the pool of endogenous free CK1δ is small due to slow synthesis and binding to the pericentrosomal region[28], we employed overexpression to analyze localization and degradation of unassembled CK1δ. Our data indicate that unassembled CK1δ is degraded in G1, coinciding with cell cycle stages in which its nuclear ubiquitin ligase, APC/C-CDH1, is active[46,47]. We show that hyperphosphorylated CK1δ accumulated in G2/M-arrested cells under both endogenous expression and overexpression conditions. Integrating our findings with previous studies, we propose that in G1 (Fig. 7), CK1δ is maintained in an active dephosphorylated state. This active CK1δ is dynamically stored at the pericentrosomal region and unassembled molecules are eliminated by the proteasome, primarily in the nucleus through APC/C-CDH1[28,29]. Because the synthesis rate of CK1δ is low and nuclear kinase is readily exported to the cytosol where it binds to the centrosome and Golgi, the fraction of CK1δ targeted for nuclear degradation is small and not detectable against higher the steady state levels. However, using CK1δ overexpression allows to further uncover this degradation pathway. In S phase, APC/C-CDH1 is inactivated by CDH1 phosphorylation and inhibition of the ubiquitin ligase by its pseudo-substrate[30,48]. Because phosphatases remain active during S phase, CK1δ is maintained in a dephosphorylated, active state. We propose that unassembled kinase is no longer degraded in the nucleus, likely allowing free kinase to support DNA repair and checkpoint control[14,17,49]. CK1 phosphorylation triggers β-TrCP-mediated degradation of MDM2 and activates p53, thereby enhancing p53-dependent responses involved in checkpoint signaling and DNA repair[50,51]. Following DNA repair, CK1δ promotes checkpoint exit by phosphorylating WEE1, leading to its β-TrCP-dependent degradation and consequent relief of CDK1 inhibition. CK1δ also supports CHK1 stability during replication stress and, together with CK1ε, phosphorylates Topo IIα to facilitate DNA decatenation in late S/G2, thereby contributing to the orderly resumption of cell-cycle progression[17,52]. Upon mitotic entry, downregulation of phosphatase activity including PP1 and PP2A[38,39], and activation of mitotic kinases[53] facilitate (auto)phosphorylation and autoinhibition of CK1δ, terminating its DNA repair functions. Because CK1δ also contributes to mitotic processes[14,17,26,54,55], a fraction of CK1δ assembled with its binding partners may remain active through locally retained phosphatase activity[43]. The majority of CK1δ, however, becomes phosphorylated and stabilized in an autoinhibited form, safeguarding it for transiently from degradation at mitotic exit, when both phosphatases and APC/C-CDH1 regain activity. At the onset of G1, reactivated CK1δ redistributes preferentially to the pericentrosomal region, while excess dephosphorylated kinase is degraded. By reactivating the safeguarded pool of CK1δ, cells can rapidly re-establish G1-specific steady-state levels without entirely replenishing the kinase through de novo synthesis, which is rather slow. We have no data regarding the order of events or their kinetics after cells exit mitosis. In particular, we do not know how rapidly CK1δ is dephosphorylated by reactivated phosphatases nor how efficiently it associates with pericentrosomal binding sites to escape degradation by reactivated APC/C-CDH1. Furthermore, our data do not address whether and how tail phosphorylation regulates CK1δ in G1. However, the bulk of CK1δ remains dephosphorylated in G1 indicating that tail phosphorylation is transient. Together, our findings reveal that the activity and abundance of dephosphorylated and phosphorylated CK1δ are regulated in a cell cycle-dependent manner (Fig. 7), suggesting distinct physiological functions associated with each phosphorylation state of the kinase. Although our

model integrates the new findings reported here together with the data on the regulation of CK1δ in G1[28] and the broader body of knowledge regarding both CK1δ biology and cell-cycle regulation, it is far from complete, and many important questions remain open. However, the model offers plausible novel concepts and mechanistic ideas that provide a basis for further investigation and discussion.

Materials and Methods

Plasmids

Plasmids were constructed as previously described[12]. Briefly, restriction digestion, dephosphorylation of vector backbone and ligation was performed as recommended by New England Biolabs (NEB). Linear DNA was purified using NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel. Plasmids were extracted with GeneJET Plasmid Miniprep kit from Thermo Fisher Scientific. For cloning with overlapping regions (restriction enzyme-free cloning), enzymes (Q5 DNA polymerase, restriction enzyme DpnI and T4 DNA polymerase) were obtained from NEB. DpnI digestion and exonuclease reaction with T4 DNA polymerase was performed in NEBuffer 2.1. *E. coli* were transformed with exonuclease treated PCR product(s) to facilitate *in vivo* plasmid assembly via overlapping DNA ends. pcDNA4/TO-derived vectors were constructed by cloning the coding regions of the human CK1δ1 splice variant, referred to as CK1δ throughout the manuscript, and CK1ε, downstream of the CMV-TetO2 promoter which allows for DOX-inducible protein expression in cells harboring the tetracycline-repressor cassette (tx; i.e., U2OStx) and constitutively high expression in cells without the tetracycline-repressor cassette (i.e., HEK293T). Mutations (CK1δ1-K38R) and C-terminal FLAG-tags were introduced via ligation-free overlap cloning. All plasmid DNA sequences were sequence-verified prior to use.

Cell culture and stable cell line generation

T-REx-U2OS (U2OStx; Life Technologies) and HEK293T (ATCC) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1x Penicillin-Streptomycin (Pen-strep). Cell culture reagents were obtained from Life Technologies. Cells were grown and maintained at 37°C in a humidified incubator containing 5% CO2.

For plasmid transfection, U2OStx cells were transfected with Xfect Transfection Reagent (Takara Bio Inc., Cat# 6313317) following the manufacturer's protocol whereas HEK293T cells were transfected with polyethylenimine (PEI, made in-house) following the conventional protocol. To generate stable U2OStx cell lines, basal U2OStx cells were seeded in a 24-well plate and grown to confluence overnight. Cells were transfected with pcDNA4/TO vectors containing coding regions of CK1δ, CK1δ-K38R, or CK1ε with C-terminal FLAG epitopes. Stable transfectants were selected by growing cells to sub-confluence in complete media supplemented with 50 μg/mL hygromycin and 100 μg/mL zeocin (Invitrogen, Cat# R25001) over the course of two to three weeks. Resulting single cell colonies after selection were isolated for further experimentation as previously described. To induce protein overexpression, cells were treated with 10 ng/mL DOX.

Immunofluorescence

U2OStx, U2OStx_CK1δ, or U2OStx_mK2-CRY1 cells were seeded on 12mm #1.0 glass coverslips (VWR, CAT# 631-1577P). Immunofluorescence was done either post-seeding or post-plasmid transfection and 24 h post DOX-induction. Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilized with PBS + 0.1% Triton X-100 for 10 min, then washed three times and blocked with PBS + 5% heat-inactivated FBS for 1 h. Cells were then treated with either mouse monoclonal anti-CK1δ (abcam ab85320, clone AF12G4, 1:5000) or mouse monoclonal anti-FLAG antibody (Sigma-Aldrich F3165, 1:200), or rabbit polyclonal anti-PCNT (abcam ab4448, generous gift from Dr. Cornelia Sala and Prof. Elmar Schiebel, 1:500), or rabbit polyclonal anti-hPER2 (in-house, 1:50) diluted in PBS + 3% BSA + 0.25% Tween20 for 1 h to detect endogenous or overexpressed CK1δ, respectively. This was followed by washing with PBS, and treatment with the corresponding secondary antibody goat anti-mouse

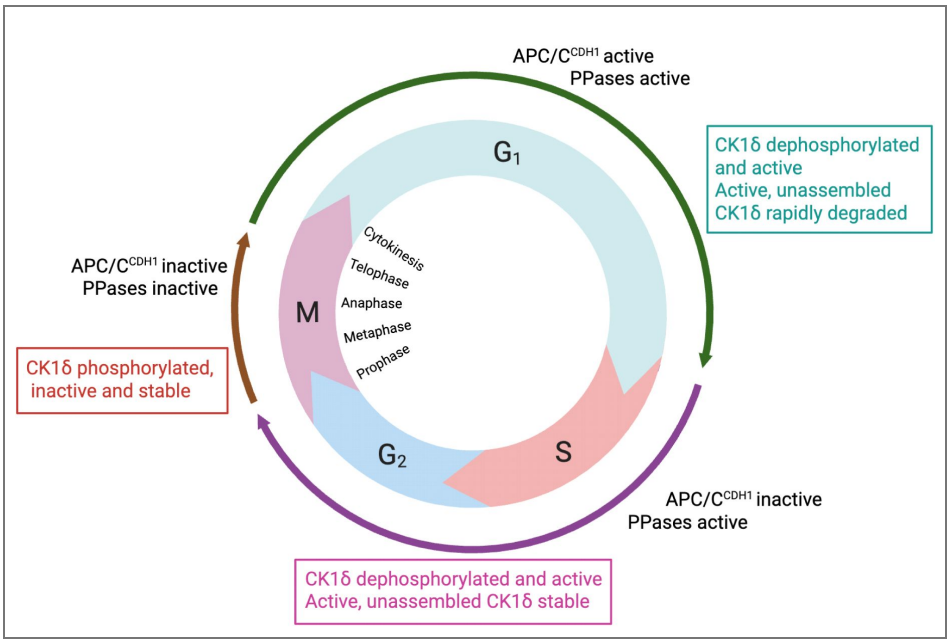

Figure 7. Model of CK1δ regulation over the course of the cell cycle

For details see main text (discussion section).

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Cell line (*Homo sapiens*)	U2OStx	Life Technologies	R712-07	
Cell line (*Homo sapiens*)	HEK293T	ATCC	CRL-3216	
Antibody	anti-CK1δ, mouse monoclonal	abcam	ab85320, AF12G4, RRID: AB_1860174	IF: 1:1000 IB: 1:5000
Antibody	anti-PCNT, rabbit polyclonal	abcam	ab4448, RRID: AB_304461, generous gift from Dr. Cornelia Sala and Prof. Elmar Schiebel	IF: 1:500
Antibody	anti-FLAG, mouse monoclonal	Sigma-Aldrich	F3165, RRID: AB_259529	IB: 1:5000 IF: 1:200
Antibody	anti-PER2, rabbit polyclonal	Pineda Antikörper-Service	Rabbit sera purified in-house	1:50
Antibody	anti-V5, mouse monoclonal	Thermo Fisher Scientific	R960. RRID: AB_2556564	1:5000
Antibody	anti-mouse IgG (H+L) conjugated to AlexaFluor™ 488, goat polyclonal	Thermo Fisher Scientific	A11001, RRID: AB_2534069	1:500
Antibody	anti-rabbit IgG (H+L) conjugated to	Thermo Fisher Scientific	A10520, RRID: AB_10563288	1:500

Key resources table.

	Cy3, goat polyclonal			
Antibody	anti-mouse IgG (H+L)-HRP conjugate, goat polyclonal	Bio-Rad	170-6516, RRID: AB_11125547	1:10000
Chemical compound, drug	Hygromycin B	Roth	CP13.4	
Chemical compound, drug	Zeocin	Life Technologies	R25001	
Chemical compound, drug	Doxycycline (DOX)	Clontech	631311	
Chemical compound, drug	Calyculin A (CalA)	LC Laboratories	C-3987	
Chemical compound, drug	Okadaic Acid (OA)	Merck	O9381	
Chemical compound, drug	PF670462	Tocris Bio-Techne	3316	
Chemical compound, drug	Cycloheximide (CHX)	PanReac AppliChem	A0879	
Chemical compound, drug	Thymidine	Merck	T9250	
Chemical compound, drug	Nocodazole	Merck	SML1665	
Recombinant DNA reagent	pcDNA4/TO	Life Technologies	V1020-20	
Commercial assay or kit	PCR Cleanup Kit	Macherey-Nagel	740609	
Commercial assay or kit	GeneJET Plasmid Extraction Kit	Thermo Fisher Scientific	K0503	
Commercial assay or kit	Xfect Transfection Reagent	Takara Bio	631317	
Commercial assay or kit	ProLong™ Glass Antifade Mountant with NucBlue	Thermo Fisher Scientific	P36983	
Software, algorithm	Fiji (ImageJ)	NIH	https://imagej.nih.gov/ij/	
Software, algorithm	GraphPad Prism 10	Graphpad Software, Inc., La Jolla, CA	http://www.graphpad.com/	
Software, algorithm	Nikon Imaging	Nikon Europe B.V,	https://www.microscope.healthc	

Key resources table. (continued)

		Amstelveen, Netherlands	are.nikon.com/en_EU/products/software/nis-elements	
Software, algorithm	BioRender	BioRender, Toronto, Ontario, Canada	https://www.biorender.com/	

Key resources table. (continued)

conjugated to AlexaFluor488 (AF488; Thermo Fisher Scientific A11001, 1:500) or goat anti-rabbit conjugated to Cy3 (Thermo Fisher Scientific A10520, 1:500) diluted in PBS + 3% BSA + 0.25% Tween20 for 1 h at 37°C. Cells were again washed three times, then mounted onto glass slides with ProLong™ Glass Antifade Mountant with NucBlue (Thermo Fisher Scientific P36983) and sealed with nail polish.

Fluorescence microscopy

For microscopy of fluorescently-tagged proteins and IF, a widefield Nikon Ni-E microscope was used (Nikon Imaging Center, Heidelberg), at a 60X magnification oil immersion objective. The corresponding filter sets for DAPI, AF488, Cy3, and mK2 were then used to visualize the specific signal from each fluorophore used. Exposure times were optimized and kept constant within each experiment to obtain comparable fluorescence intensities between treatment conditions (i.e., no treatment vs. CHX + PF670462 treatment). For image acquisition, the central focal plane was determined and images were taken as at 0.3 µm z-steps to a total 11 steps.

Digital image analysis

To analyze the resulting micrographs, built-in programs in FIJI were used. To quantify CK1δ localization at the centrosome, the PCNT channel was used to detect the subcellular localization of the centrosome. The signal intensity at the corresponding CK1δ and FLAG channels were then quantified and background-subtracted. These values were then tabulated for statistical analysis via Welch's t-test using the using the built-in analysis in GraphPad Prism 10. The resulting data was visualized using GraphPad Prism 10.

To perform the line plot analysis, a region-of-interest (ROI) was selected using the line selection tool followed by using the built-in plot profile function in FIJI. These data were then visualized using GraphPad Prism 10.

Immunoblotting

Protein extraction was performed using an in-house lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X100, 2 mM EDTA, 1 mM NaF, freshly added protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (5 µg/ml), and pepstatin A (5 µg/ml)) according to the conventional protocol. Briefly, cells were scraped and sample was collected via centrifugation. The sample was incubated in ice-cold lysis buffer on ice for 10 min and then incubated in a sonicated water bath for 10 min. Samples were centrifuged at 16,000 x g, 4 °C, 15 min taking the supernatant. Protein concentration was quantified via Nanodrop (NP80, Implen). For immunoblot analysis, protein samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (cytiva, Amersham Protran, 0.45 µm pore size) via semi-dry blotting. To control for protein loading, membranes were stained with Ponceau S. Membranes were typically incubated in primary antibody at 4°C overnight and in secondary antibody conjugated to horseradish peroxidase at room temperature for 2 h. The following primary antibodies were used: anti-FLAG (Sigma-Aldrich F3165, 1:5000, mouse monoclonal), anti-CK1δ (abcam AF12G4, 1:5000, mouse monoclonal), and the following secondary antibody was used for luminescent detection: goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad 170-6516, 1:10000). For decoration, membranes were incubated in decoration buffer (100 mM Tris-HCl pH 8.5, 2.2 x 10-2 % (w/v) luminol, 3.3 x 10-3 % (w/v) coumaric acid, 9.0 x 10-3 % (v/v) H2O2) and exposed for a time series to X-ray films (Fujifilm). Films were then developed (Medical film processor from Konica Minolta). Densitometric analysis of immunoblots was done using the built-in module in FIJI. The results were measured and plotted onto GraphPad Prism for downstream analysis (i.e., plotting densitometric signal over time post-CHX chase).

Phosphatase inhibition and cycloheximide chase

To characterize phosphatase activity, cells were treated with either CalA or OA. To inhibit phosphatases over a time course, cells were treated with 80 nM CalA (LC Labs Cat# C-3987) for 60, 20, 6, and 0 (untreated) min. All chemical treatments were performed with the corresponding

solvent (vehicle) control such as DMSO for CalA and H_2O for CHX and PF670462.

Unless otherwise stated, induction of protein expression was typically performed by addition of 10 ng/mL doxycycline (DOX; Thermo Fisher Scientific Cat# 631311) 24 h prior to downstream experiments. To arrest protein translation for a cycloheximide (CHX) chase, CHX was added to our cells to a final concentration of 10 µg/mL. Untreated samples were taken as the 0 min CHX sample. Samples were then taken at 15, 30, 45, 60, and 90 min post-CHX for protein extraction and immunoblotting. To inhibit CK1δ or CK1ε; cells were treated with 1 µM PF670462 (Tocris Biosciences Cat# 3316) for 4 h prior to immunoblotting and immunofluorescence.

Cell cycle arrest protocol

Cell cycle arrest protocol was adapted from Apraiz et al., 2017[45]. For both the S phase release and cell cycle arrest, U2OStx or U2OStx_CK1δ, were seeded onto four 10 cm dishes to 50% confluence. To release cells from S phase, a double thymidine (THY, final concentration of 2mM) was used as follows (see Fig. 5A): 24 h post-seeding, cells were treated with THY for 18 h, released into normal media for 6 h, treated with THY for a second block for 12 h. Cells were then released from the second THY block and sampled at 0, 1, and 2 h post-release.

To arrest cells in either G1/S or G2/M, either a double thymidine or a thymidine-nocodazole block, respectively, was used. For the unsynchronized sample, no additional treatment was performed (see Fig. 5C). For both the G1- and G2-arrested samples, after 24 h post-seeding, cells were treated with thymidine (THY; final concentration of 2 mM) for 18 h followed by a release into normal media for 6 h. To arrest cells in G1, cells were treated with a second thymidine block for 12 h. To arrest cells in G2, cells were treated with nocodazole (NOC, final concentration of 50 ng/mL) for 12 h. For the G2/M-arrested cells, an additional mitotic shake-off was performed to enrich the sample for cells in mid-mitosis that are loosely attached to the culture surface[44]. To induce CK1δ, DOX induction was performed on all samples 24 h prior to the end of either the double THY or THY-NOC treatments.

Material availability

Plasmids and cell lines generated in this study are available from the lead contact, Michael Brunner (*michael.brunner@bzh.uni-heidelberg.de*) with a completed materials transfer agreement.

Data availability

The data supporting the findings of this study are available within the article. This study includes no data deposited in external repositories.

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Peer reviews

Reviewer #1 (Public review):

We appreciate the authors have provided answers to many of the points we raised, and the changes made to their manuscript, which we think strengthen the overall evidence presented. However, we find that some important controls are still missing across experiments.

Major comments:

(1) Shortcomings in Immunofluorescence experiments:

a. Antibody cross-reactivity was only tested against CK1ε, but should also be tested against CK1α, which is abundant in U2OS cells, and is also known to be involved in cell-cycle regulation.

b. Fig. 1: Statistical analyses are missing from the analysis.

c. Fig. 2: No colocalisation analysis shown for figure 2, only some arrowheads pointing to puncta. Appropriate colocalisation statistics are important since for practical reasons, only a few representative images can be shown on the figure.

d. Fig. 6: Even if the figure is illustrative, it is important to show centrosome staining to visualise CK1δ's recruitment to the centrosome in G2/prophase, especially since this information is used to propose the model in figure 7.

e. For all figures: Please mention the number of independent biological replicates in the figure legends (1, 2, 6). For figure 1, if there are 3 independent biological replicates, the quantification should take all of them into account (as opposed to the data points corresponding to 10 cells), and statistics must be done appropriately, taking those independent replicates into account. Same for the colocalisation analysis in figure 2 once you include it.

(2) Shortcomings in biochemistry experiments:

On CalA control, this is not a matter of confirming that CalA treatment works in principle, but rather to confirm that CalA treatment worked in this specific replicate. Aliquots may lose potency (e.g. with freeze-thaw cycles / exposure to light), hence checking for enrichment of phospho-proteins is essential to confirm the treatment was successful in this particular instance. In the worst-case scenario, the company may have sent the wrong compound altogether! A positive and a negative control is the basis for every experiment to make meaningful interpretation. On a separate note, many experiments have control and siRNA or compound treatments on two different gels - this should be rectified as they are meaningless if different exposures have been selected for different immunoblots.

(4) As the authors mention, the kinase is not fully inactive when tail phosphorylated. Recent research has also suggested that tail-phosphorylated CK1δ may show increased catalytic activity for a few select, specific substrates, in the co-occurrence of pT220 (Cullati et al., 2022; Cullati et al., 2024). It is thus tricky to directly infer that phosphorylated CK1δ is inhibited, when no positive control for CK1δ inhibition was shown in the evidence presented. It would

be necessary to either nuance your claim or include a positive control for CK1δ inhibition. Please revise statements in the manuscript accordingly.

(5) It would be important to include statistical analyses for the immunofluorescence data in Fig. 1 and 2.

(9) The authors mentioned "In the eLife study, we show that inhibition of kinase activity by PF670462 stabilizes CK1δ and that the overexpressed kinase-dead mutant CK1δ-K38R is stable." Unfortunately, the data from biochemical analyses presented in the eLife publication is uninterpretable due to a lack of loading controls.

(10) While the data presented in Penas et al. strongly suggests a link between CK1δ stabilisation and the APC/C-Cdh1 complex, it is the only study to have shown it. Given that (1) science relies on data reproducibility and (2) your proposed model relies heavily on the relationship between CK1δ stabilisation and the APC/CCdh1 complex, it would be appropriate to include the investigations mentioned in our original comment.

https://doi.org/10.7554/eLife.112381.1.sa2

Reviewer #2 (Public review):

In this study, Serrano et al. employed a combination of cell biological and molecular approaches to investigate the localization and regulation of Casein Kinase CK1 during the cell cycle using U20S cells. They show that CK1 dynamically localizes between the centrosomes and the nucleus but can be sequestered away from the centrosomes upon overexpression of its binding partner PER2. They provide evidence that CK1 WT but not a phospho-null mutant strongly accumulates in a hyperphosphorylated form upon inhibition of phosphatases (using Calyculin and Okadaic Acid) and thus conclude that CK1 tail phosphorylation protects the kinase from degradation. Using synchronized cells, they show that CK1 accumulates unphosphorylated in S-phase (APC/Cdh1 inactive) but phosphorylated at the G2-M transition. Immunostaining shows that CK1 localizes to the centrosomes during mitosis.

The manuscript has improved overall, but some sections are still inconclusive and require clarification.

Major comments:

Figure 1 is inconclusive. CK1 nuclear staining is highly similar in untreated cells and in cells treated with CHX + PF670462. The reduction in centrosomal staining in these cells is barely significant. However, the authors draw very strong conclusions from these data sets. In panel B, the cells appear to have been fixed incorrectly, and the anti-PCNT shows a strong background signal. Not convinced that immunofluorescence is the best approach to look at protein dynamics in vivo.

In Figure 2, panel B, the authors should co-stain the centrosomes of cells that co-express CRY1 and CK1, as some of these dots may represent the centrosomes.

Figures 3B, please provide information on the non-phosphorylable CK1a mutant (it is mentioned as a variant in which all serine and threonine residues in the C-terminal tail were replaced by alanine). Specify the number of sites mutated and their exact position. Is this non-phosphorylable CK1a version catalytically active? Treatment of samples with inactivated PPase should be used as a control.

Strengths:

The authors reveal that the activity and abundance of dephosphorylated and phosphorylated CK1δ are regulated in a cell cycle-dependent manner. This suggests that these different pools

are associated with distinct physiological functions.

Weaknesses:

Unfortunately, some of the data are inconclusive, and there is no data/information linking the cell cycle regulation of CK1δ to its function during the cell cycle.

https://doi.org/10.7554/eLife.112381.1.sa1

Author response:

> ***General Statements.***

We thank all reviewers for their careful evaluation and constructive comments.

Fidel Serrano recently completed a related study on the role of CK1δ in the circadian clock which is published in eLife (*https://doi.org/10.7554/eLife.110786.1*). During these studies, we became interested in the physiological role of CK1δ autophosphorylation, whose functional significance has remained unclear.

In the present manuscript, Fidel discovered that the autophosphorylated, auto-inhibited form of CK1δ accumulates specifically during mitosis. These findings provide a physiological context for CK1δ autophosphorylation that has remained elusive for many years. They constitute the central foundation of our model, which further integrates previous findings on APC/C function and activity with the data presented in our eLife study.

Several suggestions and questions raised by the reviewers concern the regulation of CK1δ in cultured cells, which are predominantly in G1. Many of the corresponding experiments and analyses are already included in our eLife paper. We apologize that this overlap may complicate the review process. However, we are unable to publish identical datasets in both manuscripts.

We therefore briefly summarize here the findings from the eLife study that are most relevant to the present work.

- Overexpressed CK1δ is unstable, whereas CK1δ-K38R (catalytically inactive) and CK1δ-R178Q (altered specificity/activity) are comparatively stable (eLife Figs. 3A-E and 5B).

- Assembly with PER2 stabilizes overexpressed CK1δ (eLife Figs. 4A-D).

- Treatment with PF670462 similarly stabilizes the kinase (eLife Figs. 3F and 5D).

- CK1δ binding sites in the centrosomal/Golgi area are present in excess even relative to overexpressed kinase (eLife Fig. 5C).

The reviewers also raised questions about the PER2-CRY1 nuclear foci.

Thermodynamically stable/persisting nuclear foci form upon coexpression of PER2 and CRY1. They were preliminarily characterized in the eLife study (eLife Fig. 2). For the purposes of the present work, they constitute a serendipitous and convenient tool for studying interactions of PER with CK1δ. To induce foci formation, we used a stable cell line expressing DOX-inducible mK2-CRY1. mK2-CRY1 accumulates only at relatively low levels because the protein without a binding partner is intrinsically unstable, as confirmed by Western blot analysis (eLife Fig. 4C). Coexpression of PER2 stabilizes mK2-CRY1 and promotes the formation of nuclear PER2-mK2-CRY1 foci.

These foci contain elevated levels of endogenous CK1δ (eLife Fig. 2E and EV2), which accumulate gradually over a 24-hour period. This observation indicates that, at steady state, a

fraction of endogenous CK1δ is continuously degraded in the absence of overexpressed PER2. Because endogenous CK1δ is synthesized at a relatively low rate, the unstable pool is small at any given time and therefore not readily detectable in conventional cycloheximide chase assays against the kinetically stabilized steady-state background of CK1δ.

In our point-by-point response, we therefore refer the reviewers to the corresponding datasets and analyses presented in the eLife manuscript.

> ***Point-by-point description of the revisions.***
>
> ***Reviewer #1 (Evidence, reproducibility and clarity):***
>
> *Summary:*
>
> *Involved in various cellular pathways and processes, Ser/Thr kinase CK1δ is thought to be constitutively active and its tail autophosphorylation is suggested as a putative inhibitory mechanism of CK1δ kinase activity. Here, the authors investigate CK1δ's phosphorylation status, in relation to its location and dynamics throughout the cell cycle. Immunofluorescence and biochemistry studies showed that the subcellular distribution of CK1δ is dynamic and in equilibrium between centrosomal and nuclear pools. The authors argue that CK1δ phosphorylation protects it from degradation. Finally, the authors show differences in CK1δ phosphorylation status and location throughout the cell cycle. Combining their findings with the existing literature, they propose a model of CK1δ phosphorylation status, location and dynamics throughout the cell cycle.*
>
> *Major comments:*
>
> *(1) Shortcomings in Immunofluorescence experiments:*
>
> *a. Lack of a positive control for centrosomal staining, eg. PLK1 (Fig.1A,C, 2B, 6A-B). This would also allow a colocalisation analyses between CK1δ and a centrosomal marker, which would build a more convincing body of evidence in favour of centrosomal recruitment of CK1δ in baseline conditions. A lack of a negative control for CK1δ IF? Most CK1 antibodies also cross-react with other isoforms.*

As suggested, we show centrosomal staining with a well-studied centrosomal marker pericentrin (PCNT; revised Fig. 1). Consistent with what has been well-described in the literature that CK1δ localizes to the centrosome (Sillibourne et al., 2002; Greer & Rubin, 2011; Greer et al., 2014) and with our observations, we confirm that CK1δ is indeed localized at the pericentral region.

The CK1δ antibody does not crossreact, this is shown in Fig EV3A of the eLife paper.

> *b. Lack of proper image quantification (Fig.1A,C, 2B, 6A-B). To establish a centrosomal recruitment of the CK1δ pools, colocalisation of CK1δ and a centrosomal marker must be quantified using a standard colocalisation quantification method and shown appropriately. Same comments regarding the colocalisation of CK1δ with PER2/mK2-CRY1-positive foci.*

Fig. 1: Colocalization analysis with pericentrin (PCNT) as centrosomal marker is provided.

Fig. 2: The colocalization analysis of CK1δ (green) and mK-CRY1 (magenta) shows that in all PER2-untransfected cells (50 cells evaluated), CK1δ is concentrated at a single, mK2-CRY-negative spot, corresponding to the pericentrosomal region, which we have established in Figure 1. In contrast, in all PER2-transfected cells (15 cells evaluated) CK1δ localizes to mK2-CRY1 positive nuclear foci. The fields-of-view we have provided are representative of the typical phenotype of CK1δ localization in the presence or absence of PER2/mK2-CRY1-positive

foci. Here it should be noted that CRY1 foci formation is strictly dependent of co-expression with PER2, (eLife paper).

Fig. 6: This figure presents an analysis of fixed cells. Because only a small fraction of asynchronously growing cells is in mitosis at any given time, the number of cells assigned to individual mitotic stages is necessarily low (typically fewer than 10 cells per stage). The purpose of this figure is therefore primarily illustrative: to document and confirm the known subcellular localization of CK1δ during the different stages of mitosis rather than to provide a comprehensive quantitative analysis.

> *c. Lack of proper statistical analyses (Fig.1, 2, 6). As immunofluorescence constrains one to only show few images per condition at best, statistical analyses on broader image analysis data are essential to measure the significance of the changes observed on the representative images provided.*

See answer to previous question.

> *d. Lack of biological replicate numbers (Fig.1, 2, 6). Was it from 3 independent biological replicates?*

Fig.1 and 2: three independent biological replicates.

Fig.6 is just illustrative to show the already known localization of CK1δ across mitosis. The localization shown in the four panels is seen in all cells at the respective cell cycle stages.

> *e. NOTE: if available, using a confocal microscope would be best to provide optimal Z-axis resolution. This would provide you with more accurate colocalisation data, like CK1δ recruitment to the centrosome or PER2/mK2-CRY1-positive foci.*

Characterization of PER-CRY foci is published in the eLife paper in Fig. 2 and EV2.

> *(2) Shortcomings in biochemistry experiments*
>
> *Lack of loading controls in Fig.3A-C and 4A-B. Until they are added, no conclusions can be safely interpreted from the experiments. Myc is a good turnover control for CHX across all experiments. Enrichment of phospho-proteins upon CalA treatment?*

Loading controls are provided in this revision. Enrichment of phospho-proteins following CalA treatment has been demonstrated and published in numerous previous studies (Cegielska et al., 1998; Ishihara et al., 1989; Rivers et al., 1998).

> *(3) The authors infer from the literature that overexpressed CK1δ is unassembled, without checking it in any experiment. It could be that CK1δ overexpression drives overexpression of its binding partners, in which case most of overexpressed kinase could actually be assembled. Since CK1δ assembly is of great importance to the study's conclusions, it should be confirmed experimentally.*

In our eLife study, we show that overexpressed CK1δ is not fully assembled with stabilizing binding partners such as PER2. In fact, centrosomal/Golgi binding partners remain always available in excess even relative to overexpressed CK1δ, as demonstrated by the MG132-induced increase in centrosomal accumulation (eLife Fig. 5C). However, binding is dynamic and the affinities and concentrations are such that a substantial fraction of overexpressed CK1δ remains unbound and is therefore subject to degradation.

> *(4) The authors infer from the literature that phosphorylated CK1δ is inactive - which is not a given. All Western blotting experiments should include confirmed CK1δ substrates like PER2 or DVL3 to confirm that phosphorylated CK1δ is indeed inhibited. As added benefit, blotting for known CK1δ substrates will act as confirmation that the FLAG-tag on*

> *overexpressed CK1δ/ε does not impact its kinase function, and that CK1δ/ε inhibitor treatments like PHF670 have indeed worked. Furthermore, because CalA and OA inhibit many phosphatases, inferences on CK1δ/ε activity upon these treatments should be taken catiously.*

The activity of phosphorylated CK1δ is severely attenuated by autoinhibition, although the kinase is not completely inactive. This has been demonstrated repeatedly in the literature and does not require re-establishment in the present study. We previously showed this directly in a PNAS study by Marzoll et al. (*https://doi.org/10.1073/pnas.2118286119*). At some point, one must rely on established published findings unless there is compelling evidence supporting an alternative interpretation.

> *(5) Figure 1 requires further biochemical analyses to support immunofluorescence data. Western blotting analyses showing fluctuating CK1δ levels in nuclear vs. Centrosomal (https://pmc.ncbi.nlm.nih.gov/articles/PMC7618310/) fractions in the different treatment conditions would help illustrate the redistribution of CK1δ pools between nuclear and centrosomal areas. Additionally, adding a cytoplasmic fraction in each treatment condition would help visualise the amounts of unrecruited CK1δ in overexpressed conditions.*

Microscopy is a well-established and widely accepted approach for assessing subcellular localization. In many cases, it is superior to biochemical fractionation assays, which rarely yield completely pure nuclear or centrosomal fractions. The fractionation experiments suggested by the reviewer could provide additional supportive evidence, but they are not required to substantiate the conclusions presented here.

> *(6) Figure 2 requires further biochemical analyses to support immunofluorescence data showing colocalisation of CK1δ with PER2, using for example immunoprecipitation to show that pulling down PER2 also pulls down CK1δ, and vice versa. Optimally, an additional Western blotting experiment showing total / phospho-PER2 and CK1δ levels for each condition in cytoplasmic vs. nuclear fractions would consolidate the evidence from immunofluorescence and immunoprecipitation.*

Association of CK1δ with PER2 has been demonstrated extensively in numerous previous studies (Aryal et al., 2017; Narasimamurthy et al., 2018; Philpott et al., 2020; Cao et al., 2021; Marzoll et al., 2022; An et al., 2022) including our own eLife publication.

> *(7) The authors consistently interpret data showing CK1δ phosphorylation as CK1δ tail phosphorylation (p.8-11). A band shift in CK1δ signal on a Western blot does not in any way show the tail specifically is phosphorylated. Indeed, although CK1δ tail phosphorylation is widely recognised, several identified sites on other CK1δ domains, e.g.: the kinase domain, can be phosphorylated by CK1δ and other kinases. The authors' conclusions must therefore not mention tail-specific phosphorylation unless domain-specific phosphorylation is established. This could be done by comparing signal from antibodies specifically recognising known phospho-sites on the CK1δ tail against total CK1δ signal in experiments of Fig.3, 4, and 5.*

The electrophoretic mobility shift is caused by phosphorylation of the CK1δ tail. This has been demonstrated in numerous previous studies. For example, we generated a CK1δ variant in which all serine and threonine residues in the C-terminal tail were replaced by alanines. In this mutant, CK1δA, inhibition of phosphatases by CalA no longer induces an electrophoretic shift (Figure 3B).

> *(8) The use of anti-FLAG antibody in all figures looking at overexpressed CK1δ is not the most appropriate choice, as the anti-CK1δ antibody works perfectly for Western blotting and immunofluorescence studies. This adds more variables and hinders comparisons*

> *with endogenous CK1δ. If CK1δ is successfully overexpressed, wild-type levels should be negligible compared to overexpressed CK1δ levels, so the need for a FLAG antibody is not justified. Using an anti-CK1δ antibody in all experiments instead of anti-FLAG would confer them greater solidity.*

The amount of endogenous CK1δ is limited, and we therefore use endogenous detection only when it is scientifically necessary. FLAG-tagged constructs, in contrast, provide a robust and convenient experimental system. In the absence of evidence that the FLAG tag introduces artifacts or alters the observed behavior of the kinase, we do not consider it necessary to repeat these experiments using endogenous detection alone.

> *(9) The authors base themselves off a correlation between CK1δ phosphorylation status and its observed stability to establish a causal relationship between both ('phosphorylation protects the overexpressed kinase from degradation' p. 7; 'tail phosphorylation protects the overexpressed kinase from rapid degradation' p.9). However, no experiments performed suggest there is a causal relationship occurring. This could be done by looking at specific known CK1δ tail phospho-sites using phosphosite-specific antibodies. The authors could assess whether CK1δ stability is impacted when overexpressing wild-type vs. phospho-dead mutant CK1δ.*

In the eLife study, we show that inhibition of kinase activity by PF670462 stabilizes CK1δ and that the overexpressed kinase-dead mutant CK1δ-K38R is stable.

> *(10) The authors consistently link APC/CCDH1 with CK1δ's putative degradation, when none of their study touched on APC/CCDH1 activity or its involvement in CK1δ dynamics. To support these conclusions, fig.5 requires further biochemistry studies. To show APC/CCDH1's interaction with CK1δ, I suggest the authors (1) pull down CK1δ by immunoprecipitation and check for APC/CCDH1, phosphorylated CK1δ, and ubiquitin levels in enriched samples for each cell cycle stage in untreated vs. MG132-treated conditions. To consolidate this, the authors could (2) pull down CDH1 and check for phosphorylated and total CK1δ levels in pulled-down samples. Finally, they should investigate whether reducing CDH1 (3) expression (siRNA knock-down) and (4) CDH1 activity (specific E3 ligase inhibitor) have any impact on CK1δ levels at each cell cycle stage.*

These data were previously published in Penas et al. (doi: 10.1016/j.celrep.2015.03.016). For example, Fig. 5C shows that siRNA-mediated depletion of Cdh1, the G1-specific cofactor of APC/C, stabilizes overexpressed CK1δ, as well as the established APC/C-Cdh1 substrate Cyclin B1.

> *(11) Methods section lacks a subsection detailing image acquisition, including the type of microscope used (confocal vs. widefield), magnification used, whether acquisition settings were kept consistent throughout conditions / technical/biological replicates.*

Is provided in the revised manuscript.

> *(12) Methods section must include a subsection detailing immunofluorescence image analysis parameters and statistical analyses, e.g.: criteria for centrosomal localisation categories in Fig.1, criteria for categorising cells in different stages of mitosis (Fig.6), overall threshold / criteria stringency.*

Is provided in the revised manuscript.

> *Minor comments:*

The Reviewer has raised about 50 minor points, counting the individual remarks and sub-point and sub-sub points. We have addressed a number of these comments where they were

scientifically relevant or helpful for improving clarity. Most of the questions raised concern published and generally accepted data. We therefore do not believe that a point-by-point response to every individual minor remark is constructive or necessary.

> *(1) PF670 was shown to selectively inhibit CK1δ/ε over 42 common kinases (TOCRIS), however it is not well-characterised regarding the remaining 474 kinases encoded by the human genome. There is thus a possibility for PF670 inhibition overlap between CK1δ/ε and other kinases, which should be mentioned, and the authors' conclusions should he more nuanced as a result.*
>
> *(2) CHX is a protein synthesis inhibitor, which means it does not selectively target CK1δ expression, but that of every protein within the cell. This should be addressed and controlled for, if possible. A potential way to go about it would be to knock-down CK1δ using siRNA and compare untransfected controls with select post-transfection timepoints to assess the impact of inhibiting CK1δ expression on total CK1δ levels.*
>
> *(3) All unshown Western blotting replicates should be included in the supplementary materials.*
>
> *(4) Figure 1:*
>
> *a. A-B: experiment is missing PF670 alone and CHX alone conditions to control for direct effects of either compound, versus combined.*
>
> *b. A-B: in the text, please address the fact that >50% cells show unclear or no centrosomal pattern in untreated conditions.*
>
> *c. C: the authors claim that CK1δ-FLAG levels are decreased in PF670-treated conditions because PF670 inhibits excess CK1δ autophosphorylation, inducing its subsequent degradation. Before this claim is made, a proteasome inhibitor like MG132 should be included within the experiment, to show that this decrease in CK1δ expression under PF670 treatment can be rescued with MG132 treatment. This would plead in favour of excess CK1δ degradation and exclude the possibility that 4h of PF670 treatment simply reduces the rate of CK1δ expression. Should you wish to be more specific and confirm that APC/CCDH1 is responsible for CK1δ degradation, using a CDH1-specific inhibitor or siRNA-mediated knock-down of CDH1 could confirm that CK1δ degradation occurs via APC/CCDH1-mediated ubiquitination of CK1δ.*
>
> *d. C-D: experiment is missing pre-DOX induction control to confirm that CK1δ-FLAG is indeed being overexpressed.*
>
> *(5). Figure 2:*
>
> *a. A: should include non-transfected control panels to confirm the success of PER2 overexpression.*
>
> *b. B: Although observed in a preprint from the same team, there is no peer-reviewed evidence that establishes mK2-CRY1 as a reliable indicator of PER2 overexpression. 2A shows a correlation between both but does not exclude the fact that PER2 must be included in the imaging of the 2B panels, instead of using mK2-CRY1 as a proxy readout of PER2 expression and location. Appropriate analyses would then be required to show colocalisation of CK1δ with PER2 in the highlighted puncta.*
>
> *c. 'PER2-dependent foci': cannot be said of the data unless PER2 dependency has been validated in those images. Please see above point to resolve this.*
>
> *(6) Figure 3:*

a. B: the use of kinase-dead CK1δ mutant does not allow to fully separate direct autophosphorylation from phosphorylation by other kinases, unlike what the authors mention: CK1δ kinase activity may be required for the phosphorylation of certain sites by other kinases. In this case, the decrease of phosphorylation in the kinase-dead CK1δ mutant would not only result from inhibited autophosphorylation, but also from reduced phosphorylation by other kinases. Please adjust your conclusions accordingly (p.8).

b. C: poor visualisation of CK1δ overexpressed condition, especially showing critically reduced signal at the 6min CHX timepoint, compared to its kinase-dead homolog. If this change is present in every replicate performed, please address it in the text. If not, perhaps you may have to display another representative replicate in the figure.

c. The authors claim that the increased levels of overexpressed with CalA treatment suggest 'that full or partial phosphorylation of the CK1δ tail stabilizes both active and inactive forms of the kinase' (p.8). Importantly, tail phosphorylation was never shown, so this should be corrected. Additionally, it could be that CalA treatment considerably increases the rate of CK1δ expression - which would also match data in fig.4A, since CalA and CalA+PF670 treatments alone drastically increased CK1δ levels. This should be checked by pre-treating cells with CHX before applying CalN, or by treating cells with CHX and CalN simultaneously, and interpreted accordingly.

(7) Figure 4:

a. A: CK1δ levels in CHX-free controls from both 1h pre-treatments look much higher compared to untreated controls, which the authors interpret as an indication that 'most of the newly synthetised overexpressed kinase was degraded in untreated cells' (p.9). However other explanations are not explored: since loading controls are not provided, it may be that sample loading in the gel is simply off. Importantly, it is also possible that pre-treatments increased CK1δ expression before CHX application. Please make sure you touch on each

b. A-B: the authors claim that CK1δ-FLAG and CK1ε-FLAG levels are decreasing with CHX treatment because they are being degraded. Adding a panel with a proteasome inhibitor like MG132 would solidify this argument. Rescue of CK1δ/ε degradation under CHX treatment would show that the loss is indeed mediated by the UPS. Should you wish to be more specific and confirm that APC/CCDH1 is responsible for CK1δ degradation, using a CDH1-specific inhibitor or siRNA-mediated knock-down of CDH1 could confirm that CK1δ/ε degradation occurs via its ubiquitination by APC/CCDH1.

c. B, D: blot in B does not match the quantification trends in D. E.g.: 60min CHX + CalA + PF670 condition shows clearly lower CK1ε signal compared to its 0min CHX control. Please ensure the biological replicate you display on the figure is indeed representative of your results.

d. A, C: 'Hyperphosphorylated CK1δ remained stable throughout the CHX chase [...], indicating that tail phosphorylation protects the overexpressed kinase from rapid degradation' (p.9). Meanwhile this is true, unphosphorylated CK1δ in the CalA+PF670 treatment condition was also stabilised, showing that CK1δ phosphorylation may not be required for kinase stabilisation. This is an important point and should be addressed in the data interpretation. On another note - and as mentioned above -, tail phosphorylation specifically is not shown and cannot be inferred unless domain-specific phosphorylation is investigated.

e. B, D: CK1ε-FLAG levels decrease with CHX treatment compared to its baseline in the CalA+PF670 condition, which is not the case for CK1δ-FLAG (A). Thus, the data shown does not support the authors' conclusions 'CK1ε turnover is regulated in a similar manner to CK1δ'. Please address this issue and adjust your conclusions accordingly.

f. C-D: performing statistical analyses on protein band intensity in different conditions would be interesting to establish the significance of those changes.

(8) Figure 5

a. B: non-arrested controls mentioned in the text are missing from the figure.

b. B: 'the accumulated CK1δ remained dephosphorylated' (p.10). The experiment is missing important positive / negative controls of CK1δ phosphorylation status to conclude whether CK1δ remained phosphorylated or unphosphorylated across conditions. One or the other cannot be concluded from the blot as it is. Using phospho-specific antibodies may also help to visualise phosphorylation status.

c. B, D-E: blots should include CDH1 phosphorylation levels (hyperphosphorylated CDH1 is inactive), CK1δ substrates (indicators of CK1δ activity), and relevant phosphatase substrates to create a cohesive picture of changes in mitosis and support fig.7.

d. D-E: please address the differences in CK1δ profile between overexpressed and endogenous CK1δ in G2/M phase.

(9) Figure 6

The authors say: 'similar results were observed when using U2OStx cells and staining for endogenous CK1δ'. However, CK1δ's subcellular distribution pattern is different in endogenous vs. overexpressed conditions in the telophase-cytokinesis and post-mitosis stages. In the telophase-cytokinesis stage, endogenous CK1δ seem to form nuclear hotspots, while overexpressed CK1δ is more diffuse. In the post-mitosis stage, overexpressed CK1δ shows a clear polar pattern in the nuclear periphery, while endogenous CK1δ shows a diffuse pattern similar to that of overexpressed CK1δ described in fig.1 as 'unassembled' by the authors. Please address this and adjust your conclusions appropriately.

(10) Figure 7

a. The authors infer APC/CCDH1's activity levels or relationship to CK1δ from existing literature only. Since it is a central mechanism of their study, literature-only components are insufficient for a summary figure. To include these elements in the figure, the authors must include investigations of APC/CCDH1's activity levels and involvement in CK1δ degradation at different stages of the cell cycle in their study. Please refer to point 10.

b. Similar comment for CK1δ assembly status and activity levels. Please refer to point 4.

c. Similar comment for phosphatase activity levels in different stages of the cel cycle. By observing phosphorylation status in known substrates of established CK1δ phosphatases, one can easily confirm phosphatase activity levels.

d. Does not consider the fact that some results were different in endogenous vs. overexpressed CK1δ models. Please nuance your claims.

(11) Reference missing p.12 paragraph 1: 'Yet, overexpression of CK1δ consistently accelerates the circadian clock, implying that kinase availability can influence clock speed. This finding suggests that CK1δ activity may be regulated not only by catalytic

mechanisms but also by its spatial availability within the cell'. The facts stated there are not covered in the study's findings and is not referenced with a published study.

(12) Grammar mistakes / typos to report:

p.3 paragraph2: 'the kinases undergo futile cycles of phosphorylation and dephosphorylation'

p.26 Fig.1A legend: 'Endogenous CK1δ was detected by IF to'? Unfinished formulation

p.8: 'PP1 was previously suggested as a major PPase of CK1δ/ε'

p.8: 'fewer phosphorylation sites are targeted by other kinases'

Figure 4C legend: 'Overexpressed unphosphorylated CK1δ [instead of CK1ε] is degraded with a half-life of about 15 min'

Figure 4E: Ponceau staining

(13) Nomenclature inconsistencies to report:

p.4 paragraph2: 'protein PER2', then p.4 paragraph3 'PERIOD2'.

'CK1δ' used throughout the article's body text, but 'CSNK1D' is used in IF panel legends. The gene name was never introduced in the main text, nor has it been explained in the figure legends, so perhaps go for CK1δ for all mentions, including in figures.

'FOV' nomenclature is unclear, please define in the figure legend.

Figure 2B: what are the arrowheads pointing to? - please clarify in the figure legend.

Mislabelled figures: figure 3 in the text refers to figure 4 in the figure section, and figure 4 in the text to figure 3 in the figure section.

Figure 6A-B: abbreviated mitosis stages 'Pro' and 'Meta-Ana' should either be defined in the figure legend or put in full writing within the figure.

Reviewer #1 (Significance):

The paper will appeal to those working on CK1 biology, including cell cycle and circadian rhythms.

Reviewer #2 (Evidence, reproducibility and clarity):

Summary:

In this study, authors aimed to address functional links between CK1 activation, subcellular localization and protein stability, which is an important biological question. This manuscript is a follow up of a recent study by the same team, which is currently deposited at Biorxiv and a fraction of data seems to overlap, which is somewhat confusing. Overall, the concept that the stability of CK1d is dynamically controlled across the cell cycle is interesting. On the other hand, how this is functionally connected to the circadian cycle described in the previous study remains unclear.

The data presented in this study are highly preliminary and lack a number of essential controls, which weakens an otherwise interesting concept.

Major issues:

One of the main conclusions of the study is that CK1d is degraded predominantly in the nucleus while the centrosomal pool is protected from the degradation. This concept is

> *interesting but unfortunately, the experimental evidence supporting this model is very limited. Authors previously showed that neither inhibition of proteasome or treatment of cells with PF670462 significantly influenced levels of endogenous CK1d but both treatments promoted accumulation of the tagged and overexpressed FLAG-CK1d. Absence of the phenotype at the level of endogenous protein clearly raises question whether this may be just an artifact of overexpression, tagging or both combined.*

As shown in our previously published eLife study, CK1δ is synthesized at a relatively low rate from its endogenous locus. Free CK1δ continuously shuttles between the cytosol and the nucleus. Although association of CK1δ with the centrosomal/Golgi area is dynamic, nuclear export followed by binding to centrosomal/Golgi structures constitutes the major sink for the kinase at steady state. Consequently, the fraction of unbound CK1δ that is targeted for degradation in the nucleus at any given time is very small and cannot be detected in cycloheximide chase assays against the much larger background of kinase stabilized by association with centrosomal/Golgi binding sites. To reveal that CK1δ is subject to degradation at all, we had to overexpress the kinase. Under these conditions, the fraction of unbound CK1δ increases substantially, allowing nuclear degradation to be detected experimentally.

> *The statement that degradation occurs in the nucleus is based on weak data with CK1d-NES construct that was claimed to accumulate at higher levels compared to the wild type CK1d. However, that experiment used quantification of microscopic data in which two distinct regions (nucleus, cytosol) were compared, which is technically challenging. Including a reporter for normalizing for the transfection efficiency would strengthen conclusions of that experiment. Overall, quantification by immunoblotting may be more accurate.*

As suggested, we performed CHX time-course experiments with NES- and NLS-tagged CK1δ constructs followed by immunoblot analysis (shown in revised Fig. 4E).

> *Specificity of the microscopy staining was not validated Fig. 1. Confirmation of the staining specificity by RNAi or KO approaches is essential. This antibody from Abcam has been discontinued which makes it impossible to reproduce this experiment.*
>
> *The authors should therefore attempt to demonstrate localization using other available antibodies against CK1d.*

The antibody is available through Thermo Fisher in the United Kingdom: *https://www.fishersci.co.uk/shop/products/100-ul-mouse-monoclonal-af12g4-casein-kinase/13070202#*

It is specific for CK1δ and does not recognize CK1ε, as demonstrated in our eLife study.

Furthermore, FLAG-tagged CK1δ detected with anti-FLAG antibodies, as well as endogenous CK1δ detected with the commercial antibody, localize to the pericentrosomal region and relocalize upon PER2 overexpression to mCRY1-containing nuclear foci. Together, we consider these data compelling evidence for the specificity of the antibody staining.

> *The authors also failed to demonstrate that the dots represent centrosomes. To do so, they must perform co-staining with a robust centrosome marker.*

As requested, Pericentrin (PCNT) was used as a centrosomal marker and is now provided in the revised Fig. 1.

> *This would help classify approximately 50% of the cases that are currently assessed as "potential" but are in fact inconclusive.*

Centrosomal colocalization with PCNT improved the robustness of the quantification.

> *The quantification in the experiment is incorrect. Since the percentages are reported, both columns should add up to 100%. In Panel 1B, approximately 10% of the cells are missing, while in Panel 1D, there appear to be about 20% more cells.*

As indicated in our previous figure, the y-axis represents the number of cells, not percentages. We analyzed approximately 100 cells, which may have led to the misunderstanding that the values refer to percentages. As well, we have replaced this figure with a revised Figure 1 which shows that CK1δ co-localizes with PCNT as a centrosomal marker.

> *Fig. 2B shows four different fields based on which authors come to conclusion that co-expression of CRY and PER2 promotes re-localisation of CK1d to nuclear foci. This is an interesting possibility but it is hard to conclude without any quantification. What was the fraction of cells that expressed CRY and PER2 that showed this phenotype? What was the fraction of cells that did not show this phenotype although both of these proteins were expressed?*
>
> *It would help to label cells expressing PER2 either by expressing it as a fusion protein or by co-expressing a marker protein ideally from the same plasmid.*

All cells in this stable cell line express mK2-CRY1. The cells were transiently transfected with PER2, and therefore only a fraction of the cells received the PER2 expression construct. In our eLife paper, we showed that all PER2-expressing cells stabilized mK2-CRY1 and formed nuclear foci. We demonstrate here that every cell containing such nuclear foci also showed accumulation of endogenous CK1δ within these structures. We did not observe a single cell with PER2-induced nuclear foci that lacked endogenous CK1δ accumulation.

Cells that did not express PER2 were identified by the absence of nuclear foci and by low levels of mK2-CRY1, which was homogeneously distributed throughout the nucleus, as also shown in the eLife manuscript. In these cells, endogenous CK1δ was concentrated at a single discrete structure that, based on data shown in Fig. 1, corresponds to the pericentrosomal region. Under these conditions, neither CK1δ nor mK2-CRY1 was detected in nuclear foci.

> *Fig. 5 suggests that massive phosphorylation of CK1d, which is responsible for its mobility shift on SDS-PAGE, is most likely linked with mitosis. Authors should use established markers to estimate a fraction of mitotic cells in their G2/M fraction. In principle, there are two possible explanations for the doublet observed with CK1d staining. Ether CK1d exists in two pools with different phosphorylation states in mitosis, or perhaps more likely, this fraction contains G2 cells where CK1d is not yet modified and mitotic cells where CK1d is fully phosphorylated. Performing a shake-off experiment yielding a pure fraction of mitotic cells could help to distinguish between these two options.*

As described in the main text and the methods section, the fractions were prepared by mitotic shake-off to further enrich our sample for rounded cells that are loosely attached during mitosis.

> *Degradation of CK1d in telophase/cytokinesis when APC/Cdh1 becomes active is not apparent in Fig 6. The signal at mitotic spindle is missing, but there is still plenty of signal remaining in the cells. It is possible that the signal is just redistributed in the cell and the data shown do not support degradation of the protein. Authors could film cells expressing fluorescently labeled CK1d and quantify the signal during progression through mitosis and mitotic exit. The statement that "Following nuclear envelope reformation and mitotic exit, CK1d localized primarily to the single centrosome in each daughter cell" is incorrect. First, authors cannot deduce from the fixed cells whether they have just formed the nuclear envelope and exited mitosis.*

The reviewer is, of course, absolutely correct in the points raised.

First, we cannot deduce from fixed cells whether they have only recently exited mitosis. This was not our intention. We merely selected cells in G1 and referred to them as “post-mitotic,” without intending to imply that these cells had just exited mitosis. To clarify this point, we changed the previous statement:

“Following nuclear envelope reformation and mitotic exit, CK1δ localized primarily to the single centrosome in each daughter cell (Fig. 6A, 4th column)”

to:

“In G1, CK1δ localized primarily to the single centrosome (Fig. 6A, 4th column),” and replaced in column 4 of Fig. 6A and B the label “post-mitosis” with “G1.”

Furthermore, we cannot deduce from fixed cells whether, or to what extent, CK1δ is degraded upon mitotic exit. This was neither the intention nor the conclusion drawn from Fig. 6. Rather, we show in Fig. 3 that phosphorylated CK1δ is not degraded, and in Fig. 5 that CK1δ is predominantly hyperphosphorylated during mitosis, leading us to conclude that this phosphorylated pool of CK1δ is stable. In G1, CK1δ is dephosphorylated and unassembled kinase is degraded. We currently have no data regarding the kinetics of CK1δ dephosphorylation, assembly with centrosomal/Golgi structures, versus degradation of unassembled dephosphorylated CK1δ.

The data shown in Fig. 6 serve merely to illustrate the subcellular distribution of CK1δ, which is consistent with previous reports. In Fig. 7, we present a model that attempts to integrate the new findings reported here together with the data from our recent eLife paper and the broader body of knowledge regarding both CK1δ biology and cell-cycle regulation. Of course, we do not claim that this model does by no means represents a final verdict, and many important questions remain open. However, we believe that the model provides plausible novel concepts and mechanistic ideas that have not been proposed in previous publications and therefore merit publication, as they provide a basis for further investigation and discussion.

Live-cell imaging:

Live-cell imaging of fluorescently tagged CK1δ throughout mitotic progression and mitotic exit could, in principle, provide additional insight, but such experiments are technically extremely challenging. Moreover, the central idea of our model is that as much CK1δ as possible is preserved throughout the cell cycle, whereas degradation selectively targets unassembled and potentially harmful kinase. When CK1δ is expressed at physiological levels, which would require tagging the endogenous locus, the fraction of kinase degraded upon mitotic exit is expected to be very small and therefore likely below the threshold for reliable quantification by fluorescence microscopy. Similarly, although overexpressed CK1δ undergoes substantial degradation in G1, the kinase is simultaneously synthesized at a high rate. Hence, quantitative interpretation of overexpressed CK1δ levels during mitotic exit by microscopy (without CHX) would still be difficult.

Second, the images of interphase cells constantly show multiple dots (probably surrounding the centrosome), which is a pattern that likely corresponds to Golgi rather than a single centrosome.

The reviewer is correct. Indeed, CK1δ localization to the Golgi apparatus is well established in the literature. In our original wording, we did not explicitly distinguish between Golgi and centrosomal localization, which may have been somewhat misleading. In the revised version, we therefore refer more cautiously to the “pericentrosomal region” rather than strictly to the centrosome.

The data shown in Fig. 6 serve to illustrate the known subcellular distribution of CK1δ. In the model presented in Fig. 7, we attempt to integrate the new findings reported here together with the data from our eLife paper and the broader body of knowledge regarding both CK1δ biology and cell-cycle regulation.

> *It is unclear to which figure points the paragraph "Tail phosphorylation protects CK1δ/ε from degradation". I assume that one figure is missing.*

Figs. 3 and 4 were accidentally swapped, and we apologize for this error. The paragraph in question refers to Fig. 4, which shows the cycloheximide-induced degradation kinetics of

CK1δ/ε.

> *Minor points:*
>
> *CK1 kinase inhibitor PF670462 should not be named as PF670 as this causes confusion. Authors should either use the full name of the compound or just call it as CK1 inhibitor with providing details in the methods.*

PF670 has been changed to PF670462.

> *Fig. 3B is discrepant with the figure legend. Figure shows CK1e but legend says kinase dead CK1D-K38R*

The captions to Figs. 3 and 4, as well as the references to these figures in the text, are correct. However, the actual Figs. 3 and 4 were inadvertently swapped during figure assembly. We apologize for this mix-up.

> *The authors ïnterpretation of CK1 involvement in checkpoint is incorrect. The authors state that CK1 activity decreases p53 function promoting recovery, but Inuzuka et al (ref. 51) showed that inhibition of CK1 leads to this outcome.*

We thank the Reviewer for noting this mistake. We are no experts in p53 regulation, which is rather complex. CK1 decreases MDM2 stability and hence enhances p53 function.

We corrected the statement and placed it in the right context: “CK1 phosphorylation triggers β-TrCP-mediated degradation of MDM2 and activates p53, thereby enhancing p53-dependent responses involved in checkpoint signaling and DNA repair (Inuzuka et al., 2010; Winter et al., 2004). After DNA repair, CK1δ has…”

> ***Reviewer #2 (Significance):***
>
> *It is generally assumed that CK1 is constitutively active, which is likely an oversimplified view; in a physiological context, some degree of regulation can be expected. Demonstrating that there are several pools of CK1 that are differently regulated at the level of protein stability during the cell cycle would be a significant advance in our understanding of CK1 functions.*
>
> ***Reviewer #3 (Evidence, reproducibility and clarity):***
>
> *In this study, Serrano et al. employed a combination of cell biological and molecular approaches to investigate the localization and regulation of Casein Kinase CK1 during the cell cycle using U2OS cells. They show that CK1 dynamically localizes between the centrosomes and the nucleus but can be sequestered away from the centrosomes upon overexpression of its binding partner PER2. They provide evidence that CK1 strongly accumulates in a hyperphosphorylated form upon inhibition of phosphatases (using Calyculin), and thus conclude that CK1 tail phosphorylation protects the kinase from degradation. Using synchronized cells, they show that CK1 accumulates unphosphorylated in S-phase (APC/Cdh1 inactive) but phosphorylated at the G2-M transition. Immunostaining shows that CK1 localizes to the centrosomes during mitosis.*
>
> *Overall, they propose that the activity and abundance of CK1 are regulated during the cell cycle. However, this claim would require several experiments to support it.*
>
> *Major comments:*
>
> *- As presented, some of the data are inconclusive. Co-staining with a centrosomal marker is required to determine whether or not Ck1 localises to the centrosomes. A large proportion of cells exhibit "potential" (their term) centrosomal staining, so a centrosomal marker is essential before any conclusions can really be drawn.*

In our revised Figure 1, we confirm this centrosomal staining using an antibody against pericentrin (PCNT).

> *- Figures 3 and 4 have no loading controls, and these two figures have been mixed up in the text.*

The reviewer is right, we have corrected the Fig. 3 and Fig. 4 mix-up.

Loading controls are now also provided.

> *- A mobility shift on SDS-PAGE does not prove that a protein is phosphorylated. The authors should provide experimental evidence that the mobility shift is really due to phosphorylation. As they are inactivating phosphatases using CalA, it is likely the case, but they should prove it. Furthermore, the authors did not map any phosphorylation sites in this study, so they do not know whether CK1 phosphorylation occurs in the tail (as they assert) or elsewhere.*

We provide data in Fig. 3B showing that a CK1δ variant in which all serine and threonine residues in the C-terminal tail were replaced by alanines does not undergo an electrophoretic mobility shift upon CalA treatment. These results demonstrate that the CalA-induced mobility shift is caused by phosphorylation of the CK1δ C-terminal tail.

> *- The figure legends in general are limited and lack crucial information. For instance, in Figures 3C and 3D, how was the half-life of CK1 determined?*

We have adapted the figure caption:

(C) Densitometric quantification of n=3 Western blots (see A) shown as mean ± SD. Overexpressed unphosphorylated CK1δ is degraded with a half-life of about 15 min. Both CalA and CalA + PF670462 treatments stabilize the kinase. (D) Densitometric quantification of n=3 Western blots (see B) shown as mean ± SD.

> *- The CK1 regulatory model presented in Figure 7 is not supported by the data. What experimental evidence, for instance, shows that CK1 is inactive during mitosis? To make this claim the authors should directly assay its activity.*

We show that the majority of CK1δ is phosphorylated and therefore auto-inhibited during mitosis. In the original version, we referred to this fraction as inactive. In the revised manuscript, we refer to the phosphorylated kinase as auto-inhibited.

> ***Reviewer #3 (Significance):***
>
> *This study may be of interest to researchers working on cell cycle regulation.*

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