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CDK11^{p58} kinase activity is required to protect sister chromatid cohesion at centromeres in mitosis

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Running title: CDK11p58 and sister chromatid cohesion

Abstract:

The cyclin-dependent kinase CDK11^{p58} is specifically expressed at G2/M phase. CDK11^{p58} depletion leads to different cell cycle defects such as mitotic arrest, failure in centriole duplication and centrosome maturation and premature sister chromatid separation. We report that, upon CDK11 depletion, loss of sister chromatid cohesion occurs during mitosis but not during G2 phase. CDK11^{p58} depletion prevents Bub1 and Shugoshin 1 recruitment but has no effect on the dimethylation of histone H3 lysine 4 at centromeres. We also report that a construct expressing a kinase dead version of CDK11^{p58} fails to prevent CDK11 depletion-induced sister chromatid separation, showing that CDK11^{p58} kinase activity is required for protection of sister chromatid cohesion at centromeres during mitosis. Thus, CDK11^{p58} kinase activity appears to be involved in early events in the establishment of the centromere protection machinery.

Keywords:

Sister chromatid cohesion, CDK11, Centromere, Mitosis, Mammalian cells.

Abbreviations

CDK11 ^{p58}	Cyclin-dependent kinase 11, p58 isoform
CDK11 ^{p110}	Cyclin-dependent kinase 11, p110 isoform
CREST	Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly Telangiectasia
FISH	Fluorescence in situ hybridization
H2AT120P	Phosphorylated H2AT120
H3K4diM	Dimethylated H3K4
H3S10P	Phosphorylated H3S10
IRES	Internal ribosomal entry site
PSCS	Premature sister chromatids separation
SAC	Spindle assembly checkpoint
siRNA	Small interference RNA

Introduction:

At every cellular division, chromosomes must be equally segregated into each daughter cell. In order to ensure accurate segregation, sister chromatid cohesion must be maintained from its establishment during DNA replication until the metaphase-anaphase transition. Loss of sister chromatid cohesion before anaphase onset results in segregation defects that lead to aneuploidy, with significant potential deleterious consequences such as infertility, birth defects, tumorigenesis and cell death .

Sister chromatid cohesion is mediated by cohesin, a ring-shaped protein complex, loaded on chromatin in G1, stabilized upon replication fork progression and thought to embrace and hold together the two sister chromatids . In vertebrates, two distinct events account for cohesin removal during mitosis. First, in prophase, phosphorylation of the cohesin SA2 subunit by Plk1 and phosphorylation of the pro-cohesion factor Sororin by CDK1 and Aurora B occur . Sororin phosphorylation leads to its displacement from cohesin and its replacement by the anti-cohesion factor WAPL . During this step, called "prophase pathway", sister chromatid cohesion is lost on chromosome arms but remains protected at centromeres . At the metaphase-anaphase transition, when all kinetochores are attached to spindle microtubules, the satisfaction of the kinetochore attachment checkpoint (also called "SAC", for "Spindle Assembly Checkpoint") leads to the release of an active form of the separase, a proteolytic enzyme that cleaves the Scc1 subunit of the remaining centromeric cohesin . Protection of cohesion at centromeres during mitosis early stages is mediated by Shugoshin 1 (Sgo1) . Sgo1 is recruited at inner centromeres during early mitosis following Bub1-mediated phosphorylation of histone H2A at threonine 120 . SGO collaborate with protein phosphatase

2A (PP2A) which is thought to participate to protection of cohesion at centromeres by counteracting SA2 and Sororin phosphorylation . We had previously reported that depletion of histone deacetylase HDAC3 (histone deacetylase) leads to loss of protection of sister chromatid cohesion at centromeres in a separase-independent manner. HDAC3 depletion causes displacement of Sgo1 from centromeres and is correlated with forced acetylation and loss of dimethylation on Lys4 of histone H3 at centromeres .

CDK11 (previously known as PITSLRE) is a member of the $cdc2^{p34}$ related protein kinase family and is encoded by CDK11A and CDK11B genes . The CDK11 mRNA is translated into two major CDK11 proteins: a full-length 110 kDa isoform, CDK11^{p110}, and a shorter 58 kDa protein, CDK11^{p58}, corresponding to the C-terminal moiety of CDK11^{p110}. CDK11^{p110} is expressed during all stages of the cell cycle and is involved in transcription regulation and mRNA splicing . During G2 and M phases, upon activation of an internal ribosome entry site (IRES) present in the CDK11 mRNA, CDK11^{p58} is expressed and it is required for centrosome maturation and centriole duplication . CDK11 depletion by RNAi leads to premature sister chromatid separation (PSCS), a phenotype that can be rescued by the expression of a construct encoding CDK11^{p58} . The authors report that, upon CDK11 depletion, Bub1 is absent from centromeres and Sgo1 is still present but its localization relative to the CREST signal is altered. This result is puzzling since Bub1 is required to phosphorylate H2AT120 at centromeres which, in turn, recruits Sgo1 . Loss of sister chromatid cohesion may result from different events at different stages of the "cohesin cycle". For example, failure to recruit cohesin on chromatin in G1, lack of cohesin stabilization following replication fork progression in S phase or defects in the protection of cohesion at centromeres in mitosis can all lead to premature sister chromatid separation . We report that CDK11^{p58} is required for both Bub1 and Sgo1 localization at centromeres, but not for

centromere cohesion in G2, making CDK11^{p58} part of the centromere protection machinery. We also show that the CDK11^{p58} kinase activity is essential for this function.

Materials and methods:

Cell culture and RNAi:

HeLa-S3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.03% L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. G418 (1 mg/ml) was added to the culture medium of cell lines expressing an EGFP-CDK11^{p58} construct. The cells were transfected with luciferase siRNA (as a negative control) or a CDK11 siRNA (5'AGCGGCUGAAGAUGGAGAA-3') that targets the endogenous CDK11 mRNA but not the GFP-CDK11 mRNA using Hiperfect (Qiagen) as a transfection reagent. Immunofluorescence was performed 72 hours after transfection.

Plasmid constructs and establishment of stable cell lines:

The pEGFP- CDK11^{p58} wild type construct has been described before . This construct has been designed to be resistant to the siRNA used in this study. To obtain the pEGFP-CDK11^{p58}KD construct, pEGFP- CDK11^{p58} was amplified and mutated in its kinase domain (D224N) using the QuickChange II site-directed mutagenesis kit (Agilent Technologies) following manufacturer instructions with the following oligonucleotides: (5'TACTCCCGCGCCAGCCCAAAGTTACCCACCTTGAGGATGCC 3' and 5'GGCATCCTCAAGGTGGGTAACCTTGGGCTGGCGCGGGAGTA 3')

HeLa-S3 cells were transfected with the relevant plasmids using the transfection reagent Lipofectamine (Invitrogen). Stably transfected cells were selected with 1mg/ml G418 and

cloned by limiting dilution. Individual clones were expanded in selective media before being tested for EGFP-CDK11^{p58} expression by Western blotting.

Immunofluorescence:

Cells were cytocentrifugated and fixed for 20 min in 3 % paraformaldehyde in PBS (pH 7). Cells were then permeabilized in 1X PBS containing 0.1 % Triton X100 and 100 mM Glycerol. Following saturation of non-specific sites by incubation for 60 min in 5% FBS, cells were incubated overnight at 4 °C with the primary antibody and for 45 min at room temperature with the secondary antibody. DNA was stained with DAPI (0,1 µg/ml). Slides were mounted in ProLong Gold (Invitrogen) and observed by epifluorescence microscopy (Zeiss Axio Imager M2). Fluorescence quantification at centromeres was performed using Image-J software.

Antibodies:

Antibodies used in this work: mouse anti-Bub1, rabbit anti-phospho-H2A (T120) [Active Motif #39391, #39392]; rabbit anti-dimethyl-H3 (K4) [Cell signaling #9725], rabbit anti-phospho-H3 (T3) [Abcam #ab17352], rabbit anti-actin [Sigma #A5060], mouse anti H3S10P [Millipore #05-806]. Affinity-purified rabbit anti-Sgo1 was a gift from Pr Yoshinori Watanabe and rabbit anti-CDK11 antibody was previously described . Human CREST serum was a gift from Dr Isabelle Bahun-Riedinger. Alexa Fluor coupled secondary antibodies from Invitrogen and horseradish peroxydase coupled secondary antibodies from (Jackson ImmunoResearch) were used for detection.

Fluorescence In Situ Hybridization:

HeLa cells were transfected with CDK11, Scc1 or Luciferase siRNA. 48 hours after transfection, FISH experiment was performed using the procedure described by Schmitz et al.

with minor modifications. The synthetic probe used is specific for chromosome 11 centromere (5'-A488/AgGgTfTcAgAgCtGcTc, where uppercase letters are for DNA nucleotides and lowercase letters are for "locked nucleic acid" (LNA) nucleotides) and leads to three independent signals in the aneuploid HeLa-S3 cell line.

Results

Loss of sister chromatid cohesion following CDK11 depletion is not a consequence of premature mitosis exit:

HeLa-S3 cells were transfected with a siRNA that targets the CDK11 mRNA 3'end and that was successfully used to deplete CDK11 in a previous study. . Immunoblotting with a polyclonal CDK11 antibody 72 hours after CDK11 siRNA transfection shows efficient depletion of both CDK11 isoforms (Figure 1A). HeLa cells depleted of CDK11 displayed increased mitotic index (8.26% vs. 3.3% in control cells) (Figure 1B), and increased premature sister chromatid separation (51.75% vs. 4.43%) (Figure 1C), confirming the findings reported by Hu et al. .

To ensure that sister chromatid separation was not a consequence of a precocious mitotic exit followed by an accumulation of cells in anaphase, chromosome spreads from CDK11-depleted cells were labeled with an anti-phospho H3S10 antibody. H3S10 is phosphorylated during mitosis and this phosphorylation is rapidly lost upon anaphase onset . As shown on figure 1D, CDK11-depleted cells chromosomes displaying sister chromatid separation display strong H3S10P labeling, indicating that these mitotic cells have not progressed in the cell cycle beyond the metaphase to anaphase transition.

Loss of sister chromatid cohesion upon CDK11 depletion is restricted to mitosis:

Previous studies have reported that the lack of the short isoform (p58) of CDK11 is responsible for the cohesion defect observed in CDK11 depletion experiments . Since CDK11^{p58} is expressed during G2 and M stages of the cell cycle, it can be involved in the establishment of cohesion in S/G2 phase, in its maintenance during G2 phase, or in the protection of cohesion at centromeres against the prophase pathway phosphorylation waves during mitotic early stages. In order to assess whether sister chromatid cohesion was established and maintained upstream of mitosis, we examined the inter-centromere distance of sister chromatids pairs in G2 phase nuclei. HeLa-S3 cells were transfected with luc siRNA, CDK11 siRNA or Scc1 siRNA and inter-centromere distance was measured in G2 cells 48 hours after transfection. Since Scc1 is a component of the cohesin complex, its depletion prevents establishment of sister chromatid cohesion in interphase and, thus, Scc1- depleted cells are used as a positive control for loss of cohesion in G2. Chromosome 11 centromeres were labeled by FISH using a synthetic probe developed by us. Results displayed on Figure 2 show that the inter-centromere distances in CDK11 depleted G2 cells are undistinguishable from that of the mock-transfected control cells (0.86 μm vs. 0.78 μm respectively). In contrast, this distance was increased to 1.34 μm in Scc1 depleted cells. These data show that cohesion at centromere is not lost in G2 cells upon CDK11 depletion.

CDK11 is required for Bub1 and Sgo1 localization at centromeres:

Since CDK11 depleted cells do not display sister chromatid separation in G2, we assessed the presence of factors involved in the protection of cohesion at centromeres during mitosis. We focused on the main known centromere cohesion "guardian", Shugoshin, and on the elements involved in its deposition at centromeres. Human Sgo1 starts to accumulate at inner

centromeres during prophase and is retained until the onset of anaphase . Its recruitment is mediated by the mitotic checkpoint protein Bub1. Bub1 phosphorylates human histone H2A at centromeres on threonine 120 (H2AT120) and this phosphorylation is essential for Sgo1 recruitment . As the Bub1/H2A-T120/Sgo1 pathway is established in early mitosis to protect cohesion at centromeres and CDK11 is involved in cohesion beyond the G2 phase, we assessed whether CDK11 depletion could affect the Bub1/H2A-T120/Sgo1 pathway. CDK11-depleted cells were labeled with anti-Bub1, anti-Sgo1 and anti-H2AT120P antibodies. Human CREST autoimmune serum was used as a centromere marker and centromere signal intensities for the different labelings were quantified. CDK11 depletion induced an almost complete loss of Bub1 (Fig. 3A), H2AT120P (Fig. 3B) and Sgo1 (Fig. 3C) signals from centromeres in mitotic cells displaying PSCS, whereas the CREST signal was not affected. We had previously reported that HDAC3 depletion resulted in loss of H3K4 dimethylation at centromeres, lack of Sgo1 recruitment and premature sister chromatid separation . We therefore assessed whether CDK11 depletion could affect H3K4 dimethylation at centromeres. As shown on Figure 3D, separated sister chromatids in CDK11 depleted cells do not show any detectable H3K4 dimethylation defect at centromeres.

CDK11^{p58} kinase activity is required for protection of sister chromatid cohesion at centromeres:

We then asked whether CDK11^{p58} kinase activity was involved in the protection of sister chromatid cohesion. We generated two HeLa-S3 cell lines stably expressing either a wild-type GFP tagged-CDK11^{p58} or a D224N "kinase dead" version. Five silent mutations were introduced into the siRNA target sequence in order to make both GFP-tagged transgenes mRNAs resistant to the siRNA used in this study. As shown on Figure 4A, CDK11 RNA

interference in these cell lines results in the depletion of both endogenous CDK11 isoforms but not of the 90kD GFP-tagged protein expressed from the transgene. Figure 4B shows the results of rescue experiments using wild-type and "kinase dead" versions of GFP-CDK11^{p58}. Expression of the GFP-CDK11^{p58} wild-type construct in cells depleted for the endogenous CDK11 was able to rescue both the increased mitotic index (Figure 4B) and the cohesion defect phenotypes (3.5 % PSCS in mock-transfected cells vs. 2.8 % in CDK11 siRNA transfected cells, Figure 4C). In contrast, phenotype rescue was not observed in the cell line expressing the GFP-CDK11^{p58} kinase dead mutant after CDK11 depletion (4 % PSCS in mock-transfected cells vs. 28.2 % in CDK11 RNAi cells, Figure 4C). Similar results were obtained for the proportion of cells in mitosis following CDK11 depletion (Figure 4B).

Discussion:

Maintenance of sister chromatid cohesion until anaphase onset is a critical process required to prevent segregation defects which would lead to aneuploidy and, eventually, cell death or cancer. Premature sister chromatid separation may result from different causes, the most obvious of which being unscheduled mitosis exit. We assessed whether CDK11-depleted cells displaying PSCS had exit mitosis and found that separated sister chromatids were positive for the widely used mitotic marker phospho-H3S10 (Figure 1), indicating that these cells had not crossed the metaphase to anaphase border. Thus, CDK11 depletion results in a sister chromatid cohesion defect that may originate from a defect in any step of the cohesin cycle, such as cohesin loading onto chromatin in G1, sister chromatid cohesion establishment and maintenance in S and G2, or protection of cohesion during mitosis. The depletion of a cohesin subunit such as Scc1 leads to lack of cohesin loading and cohesion establishment and results in loose association of sister chromatids in G2 cells. However, CDK11-depleted cells

do not show this "loose association" phenotype in G2 (Figure 2), indicating that loss of cohesion in these cells occurs beyond the G2/M transition.

Vertebrates have evolved a mechanism, known as the "prophase pathway", by which cohesin is actively taken off chromosome arms during early mitosis stages, cohesion being protected at centromeres . The benefits of such a mechanism are not clear but it is thought that removing most of the cohesin from chromatin may allow to build up a stock of intact soluble cohesin that will be readily usable during the early steps of the next cell cycle . Individualizing condensed chromosome arms in prophase may also be a way to prevent chromatin entanglement between sister chromatids during chromosome segregation . Protection of cohesion at centromeres is mediated by the evolutionary conserved protein Shugoshin, whose association to phosphatase PP2A is thought to counteract PLK1-mediated phosphorylation of cohesin during the prophase pathway . Shugoshin is loaded on centromeres in late G2 phase and is secured at centromeres by binding histone H2A upon its phosphorylation on threonine 120 by the checkpoint kinase Bub1 . We have previously reported that depletion of the deacetylase HDAC3 results in forced acetylation and loss of dimethylation of histone H3 lysine 4 at centromeres. The loss of H3K4 dimethylation correlates with sister chromatid separation and displacement of Sgo1 from centromeres . We report that, upon CDK11 depletion, H3K4 dimethylation at centromeres is unaffected, while centromere signals corresponding to Bub1, H2AT120 and Sgo1 become barely detectable compared to a control (Figure 3). These findings indicate that CDK11 acts downstream of dimethylated H3K4 and upstream of the Bub1/H2AT120/Sgo1 axis to protect sister chromatid cohesion. In agreement with our data, a previous study on the involvement of CDK11 in sister chromatid cohesion reported that, upon CDK11 depletion, Bub1 interaction with centromeres was destabilized. However, the authors found that Sgo1, although mislocalized, was not absent from centromeres . This represents a clear discrepancy: in our study, CDK11 depletion

leads to a 90% decrease of Sgo1 signal at centromeres. A possible explanation is that Hu et al. used a shRNA expression vector and observed cells 48 hours post-transfection while we used a siRNA and looked at cells after 72 hours. The amount of time required to express the shRNA and to process it would reduce the period of time during which the cell progress into the cell cycle in the absence of CDK11. Thus, upon CDK11 depletion, Sgo1 would be first destabilized from centromeres but still detectable and, with time, it would detach from chromatin. We reported a similar phenomenon for Haspin-mediated phosphorylation of H3T3 at centromeres when Shugoshin is not present: upon Sgo1 depletion, H3T3P is first delocalized from centromeres to chromosome arms and, 96 hours post-transfection, its signal is no longer detectable .

Phosphorylation activity of centromeric kinases is not always required to their function at centromeres. For example, Bub1 kinase activity is essential for H2AT120 phosphorylation and subsequent Sgo1 recruitment, but is not required for SAC signaling in mammalian cells . We show that CDK11^{p58} kinase activity is required for its role in the protection of sister chromatid cohesion (Figure 4). Since centromere protection seems to rely on cross-talks between histone modifications (H3K4diMe, H2AT120P) and soluble factors recruited at centromeres, it was tempting to speculate that histones can be phosphorylated by CDK11^{p58}. Thus, we immunoprecipitated GFP-CDK11^{p58} from the corresponding cell line and used it in a kinase assay with bulk histones as a substrate. We did not detect any histone phosphorylation (not shown), suggesting that the relevant CDK11^{p58} substrate must be a non-histone protein. Thus, identification and characterization of this substrate represents a major goal in the understanding of the establishment of protection of sister chromatid cohesion at centromeres during mitosis.

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The authors (T.R, C.E., R.G., L.M.-J. and C.J.) declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors

Figures legends:

Figure 1. CDK11 depletion induces sister chromatid separation in mitotic cells.

(A). Immunoblot showing CDK11^{p110} and CDK11^{p58} protein levels depleted by siRNA transfection. Cells were analyzed 72 hours post- transfection. The membrane was probed with an anti-CDK11 antibody that recognizes both CDK11 isoforms. Actin was used as a loading control. Quantification of mitotic indexes (B) and PSCS (C) of HeLa cells depleted or not for CDK11. More than 100 mitotic cells per transfection were observed to quantify PSCS and the mitotic index was determined on at least 1000 cells. (D). Immunofluorescence microscopy on chromosome spreads obtained by cyto centrifugation of HeLa cells transfected as in (A), stained with CREST serum (red), anti-H3S10P (green) and DAPI. Scale bar is 10 μ m.

Figure 2. CDK11 depletion does not induce sister chromatid separation in G2.

(A). FISH on chromosome spreads in G2 HeLa cells. Cells were transfected with si-Luc, si-CDK11 or si-Scc1 and observed after 48h. The FISH probe (green) is specific for the chromosome 11 centromere. DNA was counterstained with DAPI (blue). Scale bar is 10 μ m.
(B). Measurement of the inter-centromere distance in HeLa cells transfected as in (A). Measurements were done on 100-200 sister chromatid pairs per transfection.

Figure 3. CDK11 is required for centromere targeting of factors involved in the Bub1/Sgo1 pathway. Immunofluorescence microscopy analysis on chromosome spreads obtained by

cyto centrifugation of mitotic HeLa cells 72 hours after transfection with the indicated siRNA. Cells were labeled for Bub1 (A), H2AT120 (B) and Sgo1 (C) (green). Cells were co-labeled with CREST serum (red) and DAPI (blue). Scale bar is 10 μ m. Quantification of Bub1, H2AT120 and Sgo1 signals at centromeres is shown on left panels.

(D). Immunofluorescence microscopy showing isolated chromosomes (left panel) of HeLa cell transfected 72 hours by si-Luc, si-CDK11 or si-HDAC3 and stained for H3K4diM (green), CREST (red) and DAPI (blue). White arrows point to the H3K4diM signal at centromeres. Scale bar is 1 μ m.

Figure 4. CDK11^{p58} kinase activity is required for protection of sister chromatid cohesion.

(A). Immunoblot analysis showing CDK11^{p110}, GFP-CDK11^{p58}, CDK11^{p58} and actin protein levels in HeLa-S3, GFP-CDK11p58 wild type and GFP-CDK11p58 kinase dead cell lines transfected with si-Luc or si-CDK11 and collected 72 hours after transfection and probed with anti-CDK11 antibody or an anti-actin antibody as a loading control. GFP-tagged constructs are resistant to the CDK11 siRNA. Quantification of mitotic index (B) and PSCS (C) of HeLa-S3, GFP-CDK11^{p58} wild type and GFP-CDK11^{p58} kinase dead cell lines transfected as in (A). At least 800 cells were counted for mitotic index and PSCS.

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