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Supplemental Information

Promotion and Suppression of Centriole Duplication Are Catalytically Coupled through PLK4 to Ensure Centriole Homeostasis

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Supplemental Information

Supplemental Figure 1.

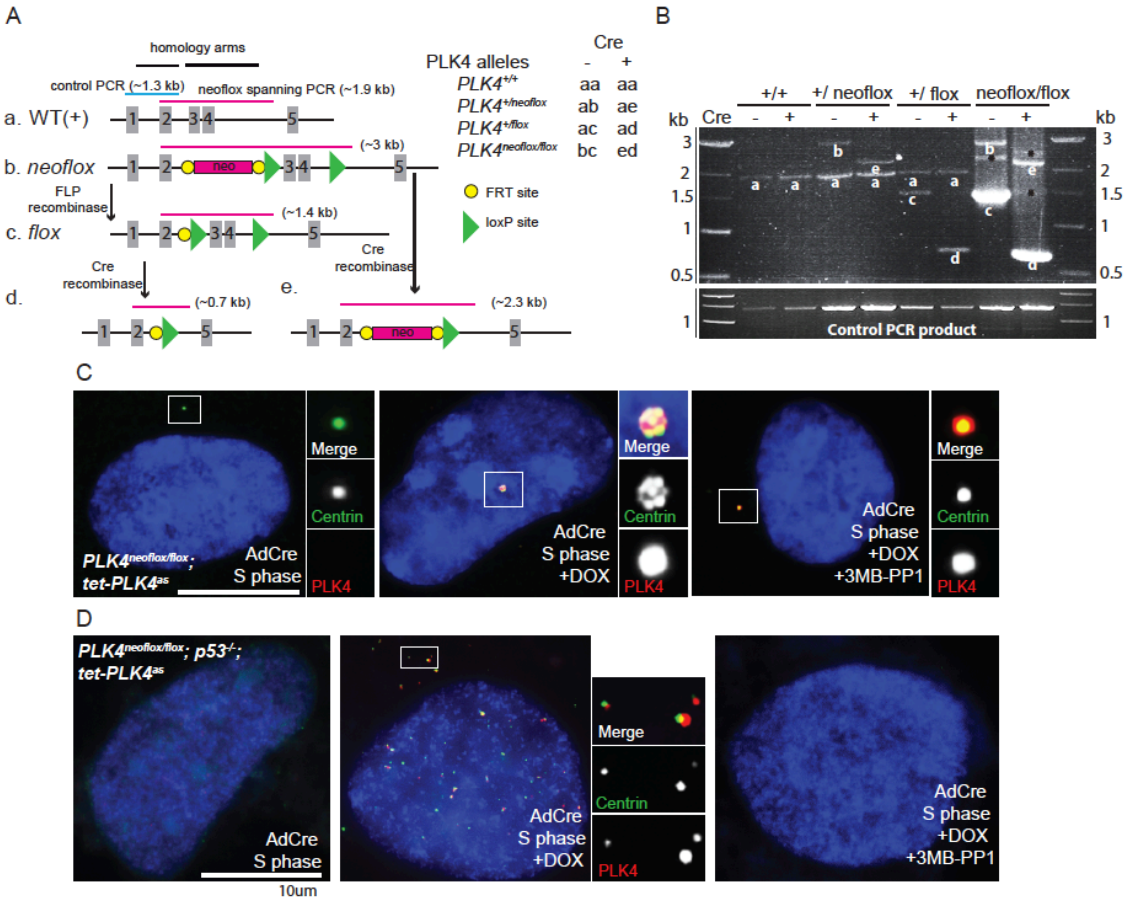

Figure S1 (Related to Figure 1). A chemical genetic system for manipulating PLK4 activity in human cells

(A) Diagram of *PLK4* locus targetd to create $PLK4^{flox/neoflox}$ alleles. *loxP* sites and *FRT* sites are indicated by triangles and circles, respectively. Wild-type, *neoflox* and *flox* alleles are detected as PCR products a, b, and c, respectively. Expected PCR products to confirm genetic manipulation are shown in magenta. Control PCR products are blue. Deletion of exon 3 and 4 from *Flox* and *neoflox* alleles by AdeCre infection give rise to PCR product d and e, respectively.

(B) PCR analysis confirming gene replacement and Cre-induced recombination in indicated *PLK4* alleles. PCR products corresponding to each alleles are indicated. Non-specific bands are noted as *.

(C) Functional test of $PLK4^{flox/neoflox}$; $tet\text{-}PLK4^{as}$ cell. AdCre infected cells were cultured for about 2 days (left), then were supplied with a high concentration of doxycycline (center), or high doxycycline with 3MB-PP1(right). Centrioles are stained with the indicated antibodies.

(D) Functional test of $PLK4^{flox/neoflox}$; $tet\text{-}PLK4^{as}$; $p53^{-/-}$ cell. AdCre infected cells were cultured for more than a week (left), then were supplied with a high concentration of doxycycline (center), or high doxycycline with 3MB-PP1(right). Centrioles are stained with the indicated antibodies.

Supplemental Table 1. Transgenic cell lines generated in this study

Name	Genetic Background	Centrosome Status	Relevant Figures
PLK4^{as}	*PLK4$^{-/-}$; tet-PLK4^{as}*	-PLK4^{as} inducible cell line propagating under low doxycycline (DOX) and carrying nearly normal numbers of centrioles/centrosomes	Figure 1 (A, C, D, and I)
PLK4^{as}; p53$^{-/-}$	*PLK4$^{-/-}$; p53$^{-/-}$; tet-PLK4^{as}*	-PLK4^{as} inducible cell line capable of losing centrioles -Acentriolar cell line in which *de novo* centriole assembly can be triggered by DOX addition	Figure 1 (B, E-H, and K)
PLK4^{as}; STIL$^{-/-}$	*PLK4$^{-/-}$; p53$^{-/-}$; tet-PLK4^{as}; STIL$^{-/-}$*	Acentriolar cell line used to reconstitute centrioles made of different forms of STILs	
PLK4^{as}; STIL^{WT}	*PLK4$^{-/-}$; p53$^{-/-}$; STIL$^{-/-}$; tet-PLK4^{as}; tet- STIL^{WT}*	-Acentriolar, PLK4^{as} cell line in which *de novo* centrioles made of STIL^{WT} can be reconstituted by DOX addition.	Figure 2 (A) and 3 (C & D)
PLK4^{as}; STIL^{4A}	*PLK4$^{-/-}$; p53$^{-/-}$; STIL$^{-/-}$; tet-PLK4^{as}; tet-STIL^{4A}*	-Acentriolar cell line with or without DOX; STIL^{4A} is not functional for centriole assembly	Figure 2 (B)
PLK4^{as}; STIL^{4D}	*PLK4$^{-/-}$; p53$^{-/-}$; STIL$^{-/-}$; tet-PLK4^{as}; tet- STIL^{4D}*	-Acentriolar, PLK4^{as} cell line in which *de novo* centrioles made of STIL^{4D} can be reconstituted by DOX addition.	Figure 2 (C) and 3 (C & D)
STIL^{WT}	*STIL$^{-/-}$; p53$^{-/-}$; tet-STIL^{WT}*	-Acentriolar in the absence of DOX -Under low DOX, cell lines with nearly normal numbers of STIL^{WT}-derived centrioles were obtained through long-term culture.	Figure 3 (E)
STIL^{4D}	*STIL$^{-/-}$; p53$^{-/-}$; tet-STIL^{4D}*	-Acentriolar in the absence of DOX. -Under low DOX, cell lines with nearly normal numbers of STIL^{4D}-derived centrioles were obtained through long-term culture.	Figure 3 (E)
PLK4^{as}KI; STIL^{WT}	*PLK4^{as}KI; p53$^{-/-}$; STIL$^{-/-}$; tet-STIL^{WT}*	-Acentriolar in the absence of DOX. -PLK4^{as} knock-in cell lines carrying nearly normal numbers of STIL^{WT}-derived centrioles were obtained through long-term culture with low DOX.	Figure 2 (D & E), Figure 3 (A, B, and F), Figure 4 (D & F)
PLK4^{as}KI; STIL^{4D}	*PLK4^{as}KI; p53$^{-/-}$; STIL$^{-/-}$; tet-STIL^{4D}*	-Acentriolar in the absence of DOX. -PLK4^{as} knock-in cell lines carrying nearly normal numbers of STIL^{4D}-derived centrioles were obtained through long-term culture with low DOX.	Figure 2 (D & E), Figure 3 (A, B, and F), Figure 4 (E & F)

Supplemental Experimental Procedures

Transgenic cell lines and plasmid constructs

PLK4$^{flox/neoflox}$ cells were generated by Adeno-associated virus (AAV)-mediated homologous recombination as described previously (Berdougo et al., 2009; Tsou et al., 2009). Template genomic DNA was from RPCI-11 human BAC clone, 398H1 (Invitrogen). Primers used in the targeting process are listed below.

PLK4 targeting primers
5'-TTTTCTCGAGCGGAAGGTGTCAGGGAGAACT-3'
5'-CCCCGATATCTGACAAATAATTCTTAATTTGCTTATGTTTAT-3'
5'-CCCCGGATCCCATTTATCTAAACCACTTAGAAAAAGAACTGG-3'
5'-
TTTTGTCGACATAACTTCGTATAGCATACATTATACGAAGTTATCCTATAAAATAGCATATTCC
TCAAAACTACAC-3'
5'-TTTTGGATCCTGGCGTCGACATATAGTAAAACTGTTTGAATTATTACTTTAAGC-3'

5’-CCCCGCTAGCATTATGAGAAATTGAGGAAGTCTGAAGC-3’

Primers to confirm gene replacement
5’-GATTTTAAAGTTGGAAATCTGCT-3’
5’-AGCAGCTTGGGATGTAGGAAATC-‘3

Control PCR primers
5’-CGGAGCGATCCATCTCGTTAC-3’
5’-TGACAAATAATTCTTAATTTGCTTATGTTTAT-3’

After generating the *PLK4*$^{flox/neoflox}$ cells, we utilized the lentiviral pLVX-Tight-Puro vector system (Clonetech) to transduce a tetracycline inducible construct expressing the analog sensitive mutant form of PLK4 (tet-PLK4^{as}). *PLK4*$^{flox/neoflox}$*; tet-PLK4*^{as} cells were then infected with adenovirus expressing Cre recombinase (AdeCre) to deplete endogenous *PLK4*, plated in 96 well plates, and cultured in the media containing 5 ng/ml of doxycycline to generate stable *PLK4*$^{-/-}$*; tet-PLK4*^{as} cell lines that carry normal number of centrosomes.

Various *STIL*$^{-/-}$*; p53*$^{-/-}$ cells inducibly expressing exogenous STIL$^{WT\ or\ 4D}$ were generated through lentiviral based transduction as described above, and isolated clonally. The targeting sequences of gRNAs used for CRISPR are as follows: p53 (5’-GGGCAGCTACGGTTTCCGTCTGG-3’) and STIL (5’-GTGTGGAATTTGACTTGCATTGG-3’).

PLK4$^{-/-}$*; tet-PLK4*^{as}*; p53*$^{-/-}$*; STIL*$^{-/-}$*; tet-STIL*$^{WT/4A/4D}$ cell lines were generated in the following order: p53 and STIL were targeted sequentially by CRISPR/Cas9 in *PLK4*$^{flox/neoflox}$; *tet-PLK4*^{as} cells. *PLK4*$^{flox/neoflox}$; *tet-PLK4*^{as}; *p53*$^{-/-}$; *STIL*$^{-/-}$ cells were infected with lentivirus carrying doxycycline-inducible STIL$^{WT,\ 4A,\ and\ 4D}$, followed by deletion of the endogenous PLK4 with AdeCre. Wild type STIL construct (Openbiosystems) and PLK4 construct (Origene RC206015) were used for subcloning into pLVX-Tight-Puro vector (Clonetech). STIL$^{4D/4A}$ (S1061D/A, S1111D/A, S1116D/A, and T1119D/A) or PLK4^{as} (L89G) mutant constructs were created with site-directed mutagenesis (Stratagene).

Cell culture
RPE1 cells were cultured in DME/F12 (1:1) medium supplemented with 10% FBS and 1% penicillin-streptomycin. *PLK4*^{as} cells (*PLK4*$^{-/-}$*; tet-PLK4*^{as}) carrying centrosomes were grown under constant supply of 5 ng/ml of doxycycline. *STIL*^{4D} and *PLK4*^{as}*KI*; *STIL*^{4D} cell lines were grown under constant supply of 20 ng/ml of doxycycline. *STIL*^{WT} and *PLK4*^{as}*KI*; *STIL*^{WT} cell lines were grown under 5 ng/ml of doxycycline. For S-phase arrest, cells were treated with 2 μg/ml aphidicolin for 12-18 hours. S-phase cells were labeled by BrdU for 30 minutes.

Assay and drug treatment
To inhibit PLK4^{as} in *PLK4*^{as} cells, excess amounts of 3MB-PP1 (20 μM, EMD Millipore) was used; to inactivate PLK4^{as} followed by reactivation, 0.2 μM of 3MB-PP1 was used to ensure proper drug washout. To inactivate endogenous PLK4, 0.2 μM of centrinone was used (Wong et al., 2015). To inhibit protein translation, 1 μg/ml of CHX was used. For reduplication assay, *PLK4*^{as}*KI* cells pretreated with 1 μg/ml CHX for 10 minutes were additionally treated with 0.2 μM of 3MB-PP1 for 1 hour. Cells were then thoroughly washed 4 times with buffers in the absence of drugs, followed by incubation in the regular medium for 2 hours.

Antibodies
Mouse monoclonal antibodies used in this study are anti-centrin2 (clone 20H5; 04-1624, Millipore), anti-SAS-6 (sc-81431, Santa Cruz Biotechnology), anti-acetylated alpha tubulin (clone 6-11B-1; T7451, Sigma-Aldrich), anti-alpha tubulin (clone DM1A, Sigma-Aldrich). A rabbit polyclonal antibody against the human C-Nap1 was produced as previously described (Tsou and Stearns, 2006). Other rabbit polyclonal antibodies used include anti-CPAP (Proteintech), anti-STIL (Bethyl Laboratories), and anti-PLK4 (kind gift from Andrew Holland at Johns Hopkins University). PLK4 and STIL antibodies were pre-adsorbed by incubating with excess amounts of methanol fixed *PLK4*$^{-/-}$; *p53*$^{-/-}$ and *STIL*$^{-/-}$; *p53*$^{-/-}$ cells, respectively to

remove non-specific materials. Rat-anti-BrdU was from AbD Serotec. Secondary antibodies Alexa-Fluor 405, 488, 594, 680 were from Molecular Probes.

Immunofluorescence and microscopy
Cells were washed once in phosphate-buffered saline (PBS) and extracted in PTEM (20 mM PIPES pH 6.8, 0.2% Triton X-100, 10 mM EGTA, 1 mM $MgCl_2$) for 2 minutes, before being fixed in methanol at -20°C for at least 10 minutes. Slides were blocked with 3% bovine serum albumin (w/v) with 0.1% Triton X-100 in PBS before incubating with primary antibodies. DNA was visualized using 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were acquired on an upright microscope (Axioimager; Carl Zeiss) equipped with 100x oil objectives, NA of 1.4, and a camera (ORCA ER; Hamamatsu Photonics). Captured images were processed with Axiovision (Carl Zeiss) and Photoshop CS6 (Adobe). For PLK4 quantification at centrosomes, all cells were treated the same during the process of immunocytochemistry and image acquisition. The images were analyzed using ImageJ software (National Institutes of Health) as described previously (Wang et al., 2011).

Super-resolution microscopy
DeltaVision OMX was employed as previously described (Fong et al., 2014).

Immunoblots
Cells were lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) supplemented with complete protease inhibitor (Roche) at 4°C for 15 minutes. Lysates were cleared by centrifugation and resolved by SDS-PAGE.

Statistical analysis
All statistical analyses were performed using Graph Pad Prism software (version 6.0). Comparisons among groups were performed by two-tailed *t*-test. Statistical significance was assigned for $p < 0.05$.

Supplemental References

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