Sufu negatively regulates both initiations of centrosome duplication and DNA replication

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Centrosome duplication and DNA replication are two pivotal events that higher eukaryotic cells use to initiate proliferation. While DNA replication is initiated through origin licensing, centrosome duplication starts with cartwheel assembly and is partly controlled by CP110. However, the upstream coordinator for both events has been, until now, a mystery. Here, we report that suppressor of fused protein (Sufu), a negative regulator of the Hedgehog (Hh) pathway playing a significant role in restricting the trafficking and function of glioma-related (Gli) proteins, acts as an upstream switch by facilitating CP110 phosphorylation by CDK2, promoting intranuclear Cdt1 degradation and excluding prereplication complex (pre-RC) components from chromosomes, independent of its canonical function in the Hh pathway. We found that Sufu localizes to both the centrosome and the nucleus and that knockout of Sufu induces abnormalities including centrosome amplification, increased nuclear size, multipolar spindle formation, and polyploidy. Serum stimulation promotes the elimination of Sufu from the centrosome by vesicle release at the ciliary tip and from the nucleus via protein degradation, which allows centrosome duplication and DNA replication to proceed. Collectively, this work reveals a mechanism through which Sufu negatively regulates the G1-S transition.

Sufu | centrosome duplication | DNA replication

For many eukaryotes, cellular proliferation requires accurate genome duplication and simultaneous centrosome duplication so that the duplicated genome can be properly distributed into the offspring cells. This genome duplication, or DNA replication, initiates during the G1-S transition through DNA licensing (1) when prereplicative complexes (pre-RC) composed of regulator proteins, including origin recognition complex 1 through 6 (ORC1-6), cell division cycle 6 (CDC6), chromatin licensing, and DNA replication factor 1 (Cdt1) and minichromosome maintenance complex 2 through 7 (MCM2-7) (2–6), assemble on DNA replication origins. Concurrently, centrosome duplication begins with cartwheel assembly (7, 8), and is partially controlled by centriolar coiled-coil protein 110 (CP110) (9, 10). RNA interference-mediated depletion of CP110 caused a centrosome duplication defect, and long-term phosphorylation ablation of CP110 by cyclin-dependent kinase 2 (CDK2) induced polyploidy (9). Some pre-RC components also participate in centrosome duplication initiation (11–15), thus indicating crosstalk between centrosome duplication and DNA replication. However, the negatively controlling regulators for both genome and centrosome duplication initiation remain unidentified.

The Hedgehog (Hh) pathway, a highly conserved pathway that is important for embryogenesis, development, and tumorigenesis (16), occurs on the primary cilium in most mammalian cells and also plays important roles in regulating cell proliferation through promoting transcription of Cyclin E and Cyclin D (17). The Hh pathway involves a number of key regulators including a 12-pass transmembrane receptor protein Patched (Ptc), a seven-pass transmembrane protein Smoothened (Smo), Suppressor of Fused (Sufu), and glioma-related (Gli) family proteins (18). The binding of Hh ligand to Ptc releases its catalytic repression on gating of Smo into the primary cilium and activation of Smo to increase the ciliary dwell time of Sufu, Gli2, and Gli3 and induces the transport of activated Gli proteins from the primary cilium to the nucleus to activate downstream transcription (19). Sufu becomes one of the key members of the Hh pathway by serving as a negative regulator. It interacts with and inhibits the Gli family of transcription factors (20), both inside and outside the primary cilium (16). Sufu also localizes in the nucleus where it is targeted by F-box and leucine-rich repeat protein 17 (Fbxl17) for proteolysis (21).

In this work, we find that Sufu localizes to the centrosome in addition to its nuclear and ciliary localization and that Sufu plays an important role in coordinating both initiations of centrosome duplication and DNA replication by serving as a negative regulator independent of the Hh pathway.

Results

Sufu Inhibits the G1-S Transition. While extending our studies on the relationships among the Hh pathway, the centrosome cycle, and the cell cycle (15, 18, 22–25), we found that in addition to ciliary and nuclear localizations, Sufu was also concentrated at one of the centrioles throughout interphase of the cell cycle (Fig. 1*A*). We also revealed that the Sufu protein level peaked during the G1-S transition and then decreased gradually until a valley in mitosis (Fig. 1*B*). However, despite the nearly equal level of cytoplasmic Sufu during the G1-S transition or in the S

Significance

The initiations of both DNA replication and centrosome duplication are strictly coordinated during the cell cycle, and failure to do so will damage the cell or induce centrosome amplification and genome instability. Here, we report that Sufu inhibits centrosome duplication via facilitating CP110 phosphorylation and suppresses DNA replication via promoting intranuclear Cdt1 degradation and expelling prereplication complex (pre-RC) components from chromosomes. Elimination of inhibitory Sufu from the centrosome counts on vesicle release at the ciliary tip, and, from the nucleus, it relies on protein degradation. This work reveals a mechanism through which Sufu, which acts as a "master switch," negatively regulates both initiations of DNA replication and centrosome duplication, and it may shed light on the preparation step control of cell proliferation.

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The authors declare no competing interest.

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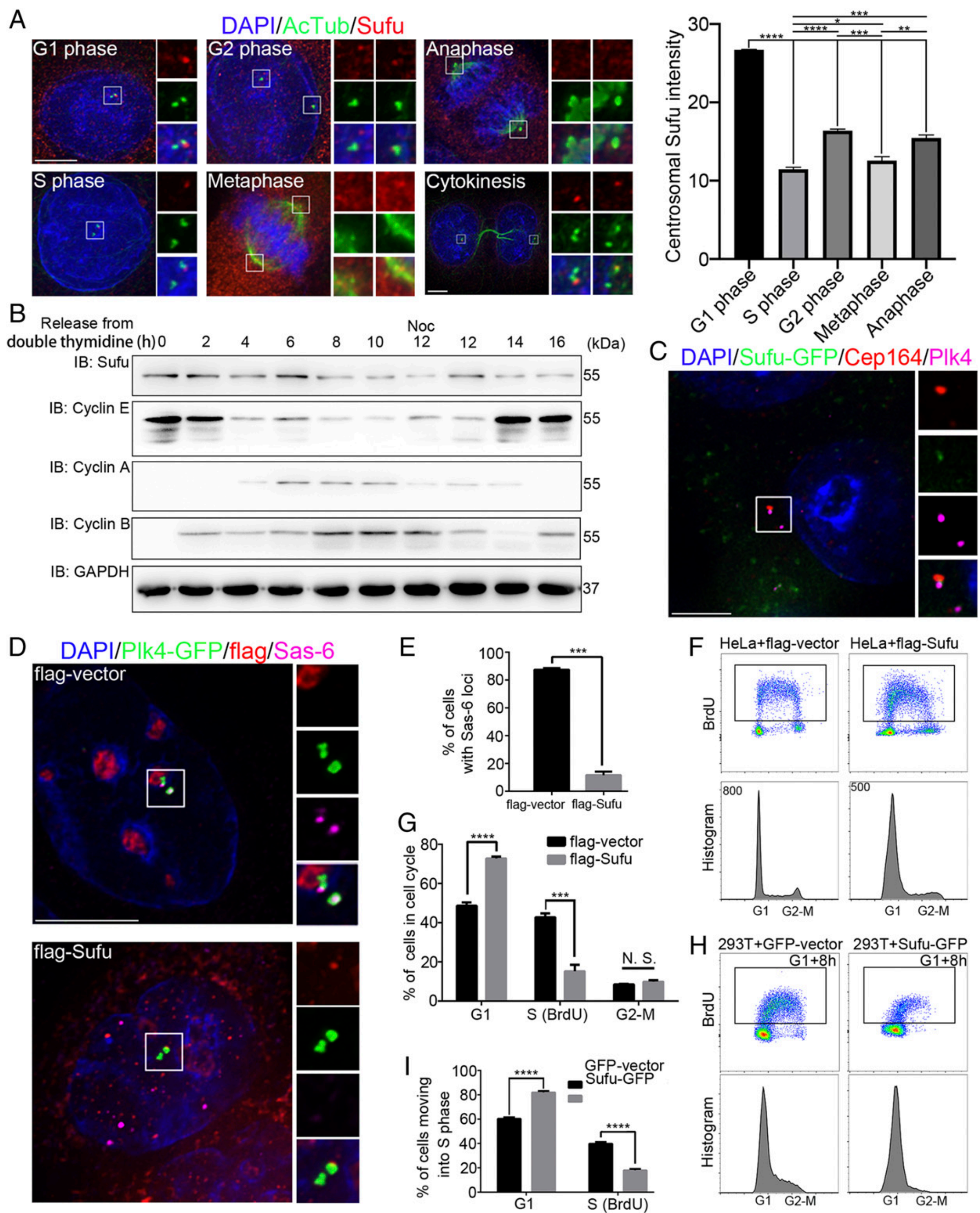

Fig. 1. Sufu negatively regulates both initiations of centrosome duplication and DNA replication in the G1 phase. (*A*) Immunostaining of U2OS cells during cell cycle with anti-Sufu and AcTub (acetylated α-tubulin) antibodies. The insets (*Right*) display magnified views of centrioles with separated channels. Fluorescence intensity was measured with ImageJ software. (*B*) Immunoblotting of synchronized U2OS cell lysates. Note the change of Sufu protein levels during the cell cycle. Cyclin E, Cyclin A, and Cyclin B1 were stained as cell cycle markers. (*C*) Immunostaining of Sufu-GFP-expressing U2OS cells with anti-Cep164 and Plk4 antibodies. The inset in the left main panel was zoomed into right panels with separated channels, displaying magnified views of Sufu colocalization at the distal end of the mother centriole. (*D*) Immunostaining of U2OS cells coexpressing Plk4-GFP with either flag-Sufu or flag-vector as control using anti- flag and Sas-6 antibodies. The insets in the left panels were zoomed in to the right panels with separated channels. Note that centrosomal Sufu expression prevented Sas-6 recruitment to the centrosome (bottom panels). (*E*) Statistic analysis of the results in *D*. (*F* and *G*) Flow cytometry cell cycle analysis of HeLa cells expressing flag-vector or flag-Sufu with BrdU labeling for DNA replication. Note that significantly more exogenous Sufu-expressing cells were in G1 and less in S (BrdU) than exogenous flag-expressing cells only. (*H* and *I*) Flow cytometry cell cycle analysis of BrdU-labeling HEK 293T cells expressing GFP or Sufu-GFP. Cells were sorted by Hoechst-stained DNA. The boxed areas in *F* and *H* are S-phase cells (BrdU); DNA was stained with DAPI (*A*, *C*, and *D*). (Scale bars, 10 μm.) For means (SD) in *A*, *E*, *F*, *G*, and *H*, 100 cells and 20,000 cells were respectively counted per replicate from three independent experiments for analysis. N. S., no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

phase, nuclear Sufu level was lower in the S phase than during the G1-S transition (*SI Appendix*, Fig. S1*A*). Furthermore, positive staining of centrosomal protein 164 (Cep164), a mature mother centriole marker, showed that Sufu-GFP, expressed at a nearly endogenous level (*SI Appendix*, Fig. S1*B*), concentrated at the distal end of the mature mother centriole (Fig. 1*C*).

To understand whether Sufu functions on the centrosomes, we first coexpressed polypeptide protein-tagged (flag)-Sufu with GFP–polo-like kinase 4 (Plk4) and immunostained Sas-6, recruitment of which to the mother centriole represents the first step of centrosome duplication (26). While the ring-like localization of GFP-Plk4 on the centrosomes was not affected, we found that overexpressing flag-Sufu significantly reduced Sas-6 centrosomal localization (Fig. 1 *D* and *E*), indicating that centrosome duplication initiation was inhibited. Flow cytometry using asynchronized HeLa cells revealed that overexpressing flag-Sufu significantly increased the percentage of G1-phase cells (Fig. 1 *F* and *G*). Analysis of isolated Sufu-overexpressing human embryonic kidney (HEK) 293T cells, which were released for 8 h from the G1-S transition, showed that excess Sufu significantly decreased the percentage of S phase entry (Fig. 1 *H* and *I*), suggesting that excess Sufu also inhibited DNA replication initiation. Collectively, these data indicate that excess Sufu inhibits initiations of both centrosome duplication and DNA replication during the G1-S transition.

Sufu Depletion Causes Multiple Defects. Interestingly, we found that using double thymidine blockage to synchronize cells to G1-S transition followed by 9 h nocodazole treatment to enrich mitotic cells, the synchronized HeLa cells expressing Sufu-GFP were hard to enter mitosis even after 12 h release from the G1-S transition (*SI Appendix*, Fig. S1*D*) and that about 40% of HeLa cells with Sufu knockdown showed centrosome amplification (*SI Appendix*, Fig. S1*E*). Sufu knockdown also dramatically downregulated the protein level of Sufu around the centrosome (*SI Appendix*, Fig. S1 *F* and *G*). Remarkably, about 60% of *Sufu*-knockout ($Sufu^{-/-}$) mouse embryonic fibroblasts (MEFs) exhibited centrosome amplification. Immunostaining of intraflagellar transport protein 88 (IFT88), a primary cilium marker, revealed that $Sufu^{-/-}$ MEFs, like wild-type (WT) MEFs, were still able to generate only one primary cilium, although the $Sufu^{-/-}$ MEFs had more centrioles (Fig. 2). These results demonstrate that knockdown or depletion of Sufu leads to centrosome amplification and that amplificated daughter centrioles were not transformed into mother centrioles.

$Sufu^{-/-}$ MEF nuclei were also significantly larger than those of WT MEFs, and many more $Sufu^{-/-}$ MEFs displayed abnormal cytokinesis (*SI Appendix*, Fig. S2 *A* and *C* and Movies S1 and S2). Meanwhile, over 40% of $Sufu^{-/-}$ MEFs died quickly after abnormal mitosis (*SI Appendix*, Fig. S2 *B* and *D* and Movie S3). Our investigation of nuclear size and cell division behavior in $Sufu^{-/-}$ MEFs revealed that the nuclear diameter in $Sufu^{-/-}$ MEFs was significantly larger than that in WT, $Ptc^{-/-}$, and $Kif3a^{-/-}$ MEFs (*SI Appendix*, Fig. S3 *A* and *B*), whereas those $Ptc^{-/-}$ and $Kif3a^{-/-}$ MEFs with more than four centrioles showed no significant difference of nuclear size (*SI Appendix*, Fig. S3 *B* and *C*). These data indicate that Sufu depletion leads to nuclear enlargement and genome instability. Furthermore, we observed that ~65% of $Sufu^{-/-}$ MEFs showed multipolar spindle assemblies (*SI Appendix*, Fig. S3 *D* and *E*), possibly a consequence of centrosome amplification or genome instability. Altogether, the results of Sufu knockdown and knockout demonstrate that a lack of Sufu causes over-duplication of centrosomes and abnormal replication of DNA.

Sufu Functions Independent of Hh Activity. Since Sufu has been comprehensively studied and shown to be a negative regulator of the Hh pathway (18–20), we wondered whether its inhibitory effects on both initiations of centrosome duplication and DNA replication are due to changes in Hh activity. To test this, we concurrently investigated centrosome duplication in $Ptc^{-/-}$, $Kif3a^{-/-}$, and $Sufu^{-/-}$ MEFs, among which $Kif3a^{-/-}$ MEFs have lost a whole primary cilium structure (27, 28). We observed that similar to $Kif3a^{-/-}$ MEFs, the percentage of $Ptc^{-/-}$ MEFs with excess centrosomes and abnormal cytokinesis was not significantly increased (Fig. 2 and *SI Appendix*, Fig. S3*B*), compared with WT MEFs, although $Ptc^{-/-}$ MEFs had higher levels of Hh activity and stronger Gli1 expression than the WT MEFs (Fig. 3*A*). In comparison, $Sufu^{-/-}$ MEFs exhibited even higher Gli1 protein levels than $Ptc^{-/-}$ MEFs (Fig. 3*A*). These results suggested that centrosome amplification in $Sufu^{-/-}$ MEFs is independent of high Hh activity and primary cilium. Then, we wondered whether the extremely high level of Hh activity in $Sufu^{-/-}$ MEFs is the reason for centrosome amplification. We treated both WT and $Sufu^{-/-}$ MEFs with GANT61, which restricts Gli1 activity (29), and observed no significant differences in the number of centrioles, size of nucleus, and percentage of multipolar spindle no matter with or without treatment of GANT61 (Fig. 3*C* and *SI Appendix*, Fig. S3 *A*, *B*, *D*, and *E*), indicating that an extremely high level of Hh activity is not the cause for centrosome amplification in $Sufu^{-/-}$ MEFs. Based on these results, we conclude that the inhibitory effect of Sufu on centrosome duplication initiation is independent of changes in the Hh activation level.

Sufu Facilitates CP110 Phosphorylation by CDK2. To find how Sufu restrains the initiation of centrosome duplication, we treated HeLa cells with hydroxyurea (HU), which efficiently induces centrosome amplification, and simultaneously overexpressed flag-Sufu in those cells. We found that the introduction of flag-Sufu undoubtedly inhibited centrosome amplification (Fig. 3*D*). We also found that flag-Sufu expression in $Sufu^{-/-}$ MEFs dramatically declined the percentage of the MEFs with centrosome amplification, resulting in that most of these MEFs possessed only two centrioles (Fig. 3*E*), indicating that Sufu strongly fixed centrosome amplification.

Upon investigating Hh activity of $Sufu^{-/-}$ MEFs, we found that CP110 had a band shift in both HeLa cells and MEFs (Fig. 3*A* and *SI Appendix*, Fig. S1*A*) and confirmed that CP110 was phosphorylated in WT MEFs but not in $Sufu^{-/-}$ MEFs via λ protein phosphatase (λ-PPase) assay (Fig. 3*B*). Since two kinases, CDK2 (9) and Plk4 (26), are reportedly able to phosphorylate CP110, and given our finding that CDK2, but not Plk4, could bind Sufu (see Fig. 5*D*), we turned our sights to the relationships among Sufu, CDK2, and CP110. Immunoprecipitation (IP) revealed that a protein complex comprised of Sufu, CP110, and CDK2 (Fig. 4*A*). Furthermore, we found that endogenous CP110 could be pulled down with CDK2-flag in WT MEFs but not in $Sufu^{-/-}$ MEFs (Fig. 4*B*) and binding of Sufu with CP110 occurred primarily during the G1-S transition (Fig. 4*C*). With the help of GFP-Plk4 as a centrosome marker, we identified Sufu, CDK2, and CP110 colocalized at the centrosome (Fig. 4*D*). To understand whether Sufu regulates centrosome duplication through mediating CP110 phosphorylation by CDK2, we generated a GFP-tagged phosphorylation mimic (by CDK2) mutant CP110-8D (9) and expressed it in $Sufu^{-/-}$ MEFs, and the results showed that the abnormal centrosome amplification was fixed (Fig. 4*E*). On the contrary, phosphorylation ablation-mimic mutant CP110-8A had an ability to induce centrosome amplification quickly before inducing polyploidy with long time ablation (9), whereas CP110-8D reduced the centrosome duplication velocity (*SI Appendix*, Fig. S3*E*). Since protein kinase A (PKA) and GSK3β have been reported to phosphorylate *drosophila* Sufu to stabilize Sufu from Hh-induced degradation (30), we tested whether PKA (which phosphorylates Sufu at S346) and GSK3β (which phosphorylates Sufu at S342) are involved in regulating

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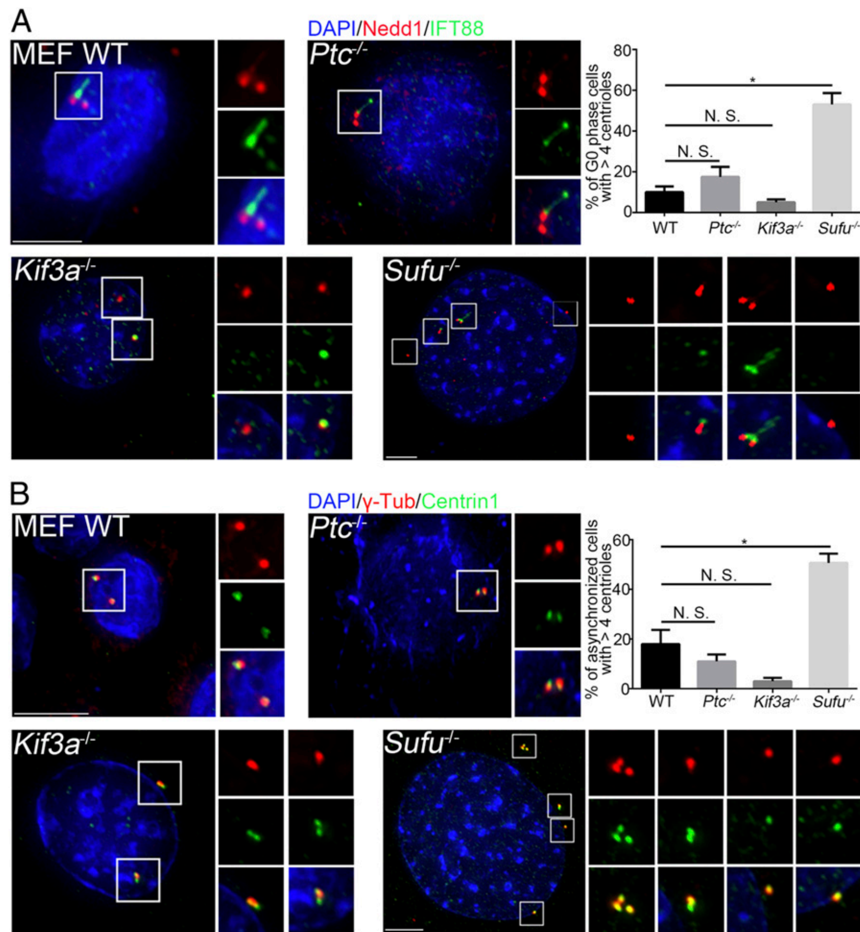

Fig. 2. Knocking out *Sufu* results in centrosome overamplification. (*A*) Immunostaining of MEFs with anti-Nedd1 and IFT88 antibodies. Boxes (*Right*) show magnified areas with separated channels of the insets (*Left*). Note that $Sufu^{-/-}$ MEFs possess significant centrosome amplification with only one primary cilium, indicated by Nedd1- and IFT88-positive staining, respectively. (*B*) Immunostaining of MEFs with anti-γ-Tubulin (γ-Tub) and Centrin1 antibodies. Boxes (*Right*) show magnified areas with separated channels of the insets (*Left*). Note that $Sufu^{-/-}$ MEFs possess significant centrosome amplification, indicated by γ-Tub-positive staining. DNA was stained with DAPI. (Scale bars, 10 μm.) For means (SD), 100 cells were counted for each replicate, and three independent experiments for analysis were carried out.

centrosome duplication. Through point mutation and mutant expression, we found that both Sufu-S346D and Sufu-S342D/S346D but not Sufu-S342D mutants exhibited raised ratio of centrosome amplification (*SI Appendix*, Fig. S3 *G* and *H*), suggesting that phosphorylation of Sufu by PKA but not by GSK3β suppresses the inhibitory function of Sufu on centrosome duplication initiation. Taken together, we conclude that phosphorylation of CP110 by CDK2 is sufficient to inhibit abnormal centrosome amplification and Sufu promotes binding of CP110 with CDK2 for its phosphorylation and inhibition on centrosome amplification, in which PKA functions in an antagonistic way via phosphorylating Sufu.

Sufu Expels Intranuclear Pre-RC Proteins. Next, due to the result that nuclear Sufu is down-regulated in the S phase (*SI Appendix*, Fig. S1*A*), we investigated the functions of intranuclear Sufu. We found that knocking out *Sufu* in MEFs remarkably increased the percentage of polyploidy and G2-M arrest (*SI Appendix*, Fig. S4*A*). We also examined whether GANT61 could induce a change of the defects and found no significant difference between WT and $Sufu^{-/-}$ MEFs in terms of cell cycle distribution and numbers of centromeres under the treatment of GANT61 (*SI Appendix*, Fig. S4 *A* and *B*). We also treated HeLa cells (Hh-deficient) with GANT61, and the results showed no difference with nontreated cells neither (*SI Appendix*, Fig. S4*C*). These results indicate that the inhibitory function of Sufu on DNA replication is also independent of high Hh activity. To further detect whether genome instability in $Sufu^{-/-}$ MEF was due to senility, we examined both levels of p16 and β-galactosidase (β-Gal), and the essentially same low level of p16 and negative staining of β-Gal indicated the exclusion of senility as a reason of genome instability in $Sufu^{-/-}$ MEFs (*SI Appendix*, Fig. S4 *D–F*).

To investigating how Sufu inhibits DNA replication, we cotransfected HeLa cells with Sufu-GFP and Cdt1-mKate2 and found that Sufu localized to both the nucleus and the cytoplasm early in the G1 phase. During ∼3 h after generation of the daughter cells, the intranuclear Sufu protein level gradually declined until it was lower than the level of cytoplasmic Sufu. More interestingly, the level of intranuclear Cdt1 was up-regulated immediately after intranuclear Sufu was reduced. After persisting for several hours and soon after intranuclear Sufu increased, intranuclear Cdt1 decreased (Fig. 5*A* and Movie S4). These results suggest that Sufu suppresses DNA replication initiation by affecting DNA licensing during the G1 phase. To verify this hypothesis, we overexpressed flag-Sufu, followed by a nucleocytoplasmic fractionation assay (NFA), and the result revealed that the pre-RC proteins CDC6, Cdt1, and MCM2-7 were all primarily retained in the cytoplasm from the nucleus (Fig. 5*B*). In

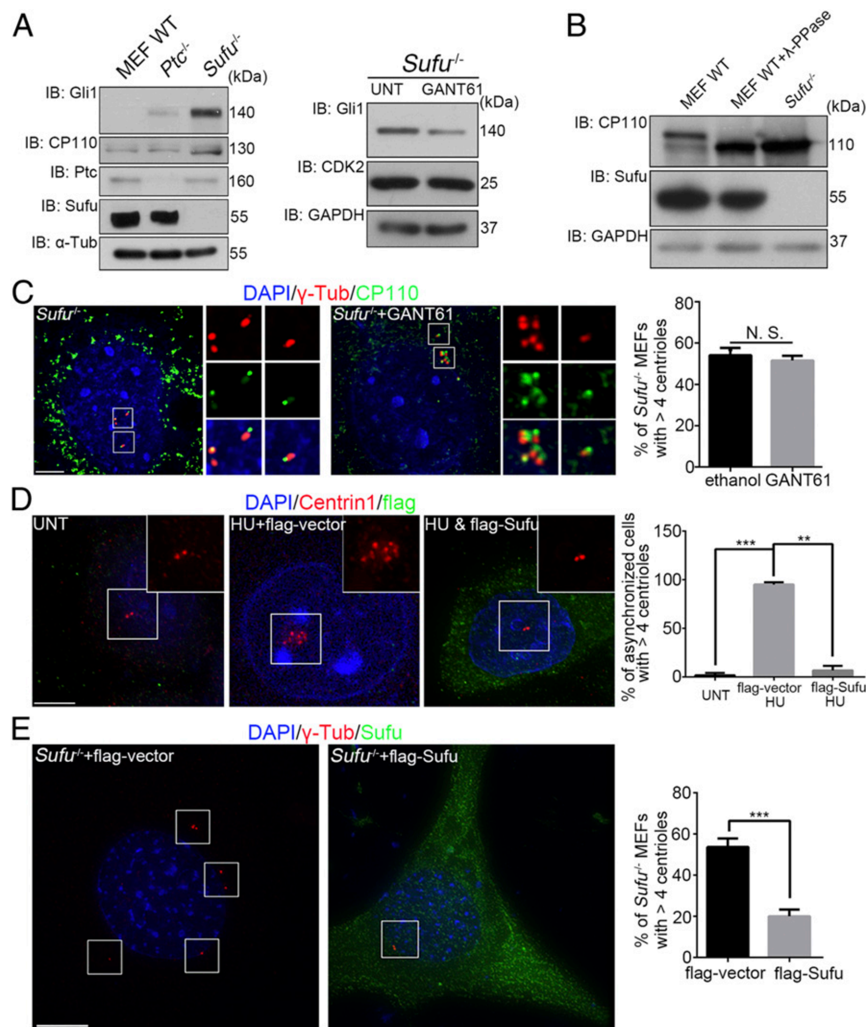

Fig. 3. Sufu negatively regulates centrosome duplication independently of Hh pathway activation. (*A*) Immunoblotting of MEF cell lysates. Note that although both *Ptc*$^{-/-}$ and *Sufu*$^{-/-}$ MEFs exhibited substantially up-regulated Gli1, indicating Hh activation, *Sufu*$^{-/-}$ MEFs showed higher levels of Gli1 and down-regulated shift position of CP110 (left panel) and that GANT61-treated Sufu$^{-/-}$ MEFs had decreased Gli1 protein levels, indicating sufficiency of GANT61 treatment in inhibiting Hh activity (right panel). (*B*) Immunoblotting of cell lysates from WT and *Sufu*$^{-/-}$ MEFs. Note that CP110 in WT but not in *Sufu*$^{-/-}$ MEFs was up-shifted and that the up-shifted CP110 band was down-shifted to the level of that in *Sufu*$^{-/-}$ MEFs when WT MEF lysates were treated with λ-PPase. (*C*) Immunostaining of GANT61-treated *Sufu*$^{-/-}$ MEFs using anti-γ-Tub and CP110 antibodies. Note that no difference between GANT61-treatment and non-treatment groups in percentages of *Sufu*$^{-/-}$ MEFs with more than four centrioles in the statistical graph. (*D*) Sufu inhibits HU-induced centrosome amplification. Immunostaining of HU-treated asynchronous flag-Sufu-expressing HeLa cells using anti-Centrin1 and -flag antibodies. Nonexpressing control (UNT) had no treatment or flag expression. The centrosome numbers were counted with Centrin1-positive staining. Percentages of HeLa cells with more than four centrioles in different groups are shown in the right graph. The number of centrioles in HU-treated flag-expressing cells was greater than in the UNT cells but significantly lower than in HU-treated flag-Sufu–overexpressing cells. (*E*) Immunostaining of flag- or flag-Sufu–expressing *Sufu*$^{-/-}$ MEFs using anti-γ-Tub and Sufu antibodies. The graph on the right shows the percentages of MEFS with more than four centrioles in distinct groups. DNA was stained with DAPI (*C*–*E*). (Scale bars, 10 μm.) For means (SD), 100 cells were counted per replicate, and three independent experiments for analysis were carried out.

addition, chromatin association assay (CAA) revealed that nuclear CDC6, Cdt1, MCM2, and MCM6 had substantial attenuation association with chromatin in the presence of excess Sufu (Fig. 5*C*), while other pre-RC components had minor attenuations, indicating that the pre-RC was not efficiently assembled in the cells overexpressing flag-Sufu. Next, we examined whether the pre-RC proteins are in association with Sufu. Through expressing Sufu-GFP followed by an IP assay, we found that MCM3, MCM4, and MCM5 could bind Sufu (Fig. 5*D*), suggesting that Sufu prevents the association of MCM3-5 with chromatin for pre-RC assembly in the nucleus and also sequesters them in the cytoplasm (6, 31). Afterward, we performed NFA and CAA in synchronized S phase cells and found that up-regulation of Sufu resulted in less association between pre-RC components and the chromatin, especially MCM2, MCM4, and MCM5 (*SI Appendix*, Fig. S5*A*), and less distribution of pre-RC components in the nuclear fraction, particularly MCM2, MCM4, MCM6, and MCM7 (*SI Appendix*, Fig. S5*C*). Endogenous Sufu could also be positively labeled in the nucleus in S phase cells (*SI Appendix*, Fig. S5*B*). We then used *Sufu* knockdown cells to examine the DNA replication rate via DNA fiber assay and revealed that decreasing endogenous Sufu significantly enhanced DNA replication rate, and this rate could be suppressed by overexpressing Sufu (*SI Appendix*, Fig. S5*D*). In contrast, when we attached a nuclear localization sequence (NLS) to Sufu-mKate2, we found that this fusion protein distributed in the nucleus the whole time. Those NLS-Sufu-mKate2–expressing or Sufu-GFP nucleus-retained cells did not pass through the G1-S transition and died thereafter (*SI Appendix*, Fig. S6 *A* and *B* and Movies S5 and S6). Collectively, these data demonstrate that

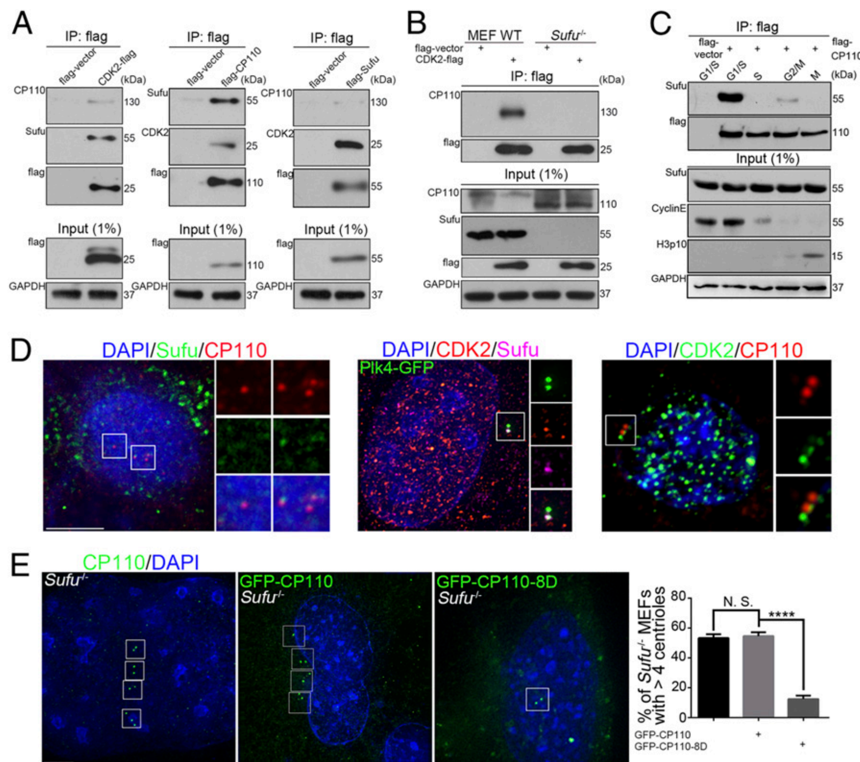

Fig. 4. Sufu facilitates CP110 phosphorylation by CDK2. (*A*) IP and immunoblotting of HEK 293T cell lysates expressing flag-tagged proteins, showing interactions among Sufu, CDK2, and CP110. (*B*) Immunoblotting of cell lysates from flag-vector– or CDK2-flag–expressing $Sufu^{-/-}$ MEFs, showing Sufu knockout disrupts the interaction between CP110 and CDK2. (*C*) IP and immunoblotting of synchronized flag-vector– or flag-CP110–expressing HEK 293T cell lysates, showing strongest interaction between Sufu and CP110 during the G1-S transition. (*D*) Immunostaining of U2OS cells using anti-CP110, Sufu, and CDK2 antibodies. Boxes in the right are magnified areas of the left main image insets with separated channels. Plk4-GFP expression in the middle panel was used as a centrosome indicator. (*E*) Expressions of GFP-CP110-WT and GFP-CP110-8D (phospho-mimetic CP110 by CDK2) in $Sufu^{-/-}$ MEFs. Note that percentage of GFP-CP110-8D–expressing $Sufu^{-/-}$ MEFs with more than four centrioles is much lower than $Sufu^{-/-}$ and GFP-CP110-WT–expressing $Sufu^{-/-}$ MEFs. DNA was stained with DAPI (*D* and *E*). (Scale bars, 10 μm.) For means (SD), 100 cells were counted per replicate, and three independent experiments for analysis were carried out.

Sufu negatively regulates DNA replication initiation by preventing chromatin association of pre-RC components for pre-RC assembly.

Nuclear Sufu Promotes Intranuclear Cdt1 Degradation. In addition to facilitating phosphorylating CP110 by CDK2 and thereby inhibiting centrosome duplication initiation (Fig. 4), Sufu may also suppress DNA replication initiation by facilitating phosphorylating Cdt1 by CDK2, which promotes Cdt1 degradation (32). To verify this hypothesis, we detected Cdt1 protein level with the treatment of MG132, which inhibits proteasomal degradation, and found that knocking down Sufu promoted Cdt1 expression, whereas overexpressing Sufu contributed to Cdt1 degradation (Fig. 6*A*). Then, we generated Cdt1-T29A mutant, which was reported not able to bind with E3 ubiquitin ligase Skp2 (33). We found that in contrast to Cdt1 WT (Fig. 5*A*), the nuclear level of Cdt1-T29A was increased immediately after cytokinesis, although the nuclear Sufu level was still high (Fig. 6*B* and Movie S7). Knowing that Sufu did not bind with Skp2 (Fig. 5*D*), we then measured binding abilities of Cdt1 WT and Cdt1-T29A with CDK2 or Skp2 through IP, and the results revealed that when Sufu was knocked down, both Cdt1 WT and Cdt1-T29A almost lost their binding abilities to CDK2, except for that Cdt1-T29A kept its weak binding ability with Skp2 (Fig. 6 *C* and *D*, left four lanes). In contrast, under over-expression of Sufu, both Cdt1-WT and Cdt1-T29A had a stronger binding ability with CDK2, and Cdt1-WT showed even higher binding ability with Skp2, whereas Cdt1-T29A was still unable to bind with Skp2 like Cdt1 WT (Fig. 6 *C* and *D*, right four lanes). These results indicate that down-regulation of Sufu reduces binding between Cdt1 and CDK2 or Skp2, whereas up-regulation of Sufu promotes the binding, and that Cdt1-T29A almost loses binding ability with Skp2, which is consistent with what was reported (33). Meanwhile, when NLS-Sufu-mKate2 was expressed in HeLa cells, the nuclear Cdt1 level significantly decreased; in contrast, when a nuclear export sequence (NES) was fused to Sufu-mKate2 (NES-Sufu-mKate2), we found that NES-Sufu-mKate2–expressing cells dramatically decreased their nuclear Cdt1 level (*SI Appendix*, Fig. S6*C*). Collectively, we conclude that nuclear Sufu promotes nuclear Cdt1 degradation through enhancing Cdt1 binding with CDK2 for phosphorylation followed by degradation.

Ciliary Scission Lifts Inhibition of Sufu on Centrosome Duplication Initiation. Knowing that intranuclear Sufu is degraded by Fbxl17 (21) and this lifts inhibition of intranuclear Sufu on DNA replication initiation, we set to investigate how cells discharge inhibitory Sufu on the centrosomes. Through expressing GFP-tagged Smo (Smo-GFP) to mark primary cilium and adding serum to stimulate cell proliferation, we found that while 20 min of serum stimulation was insufficient to trigger translocation of Smo into the primary cilium, 50% of the cells already accumulated Sufu at their ciliary tip. In contrast, when cells were treated with a small molecule Smo agonist, SAG, for 6 h, more than 90% of the cells displayed ciliary accumulation of Sufu and Smo that formed particle-like structures at the ciliary tip (Fig. 7 *A*–*C*). This is consistent with a report that Sufu can be recruited from the

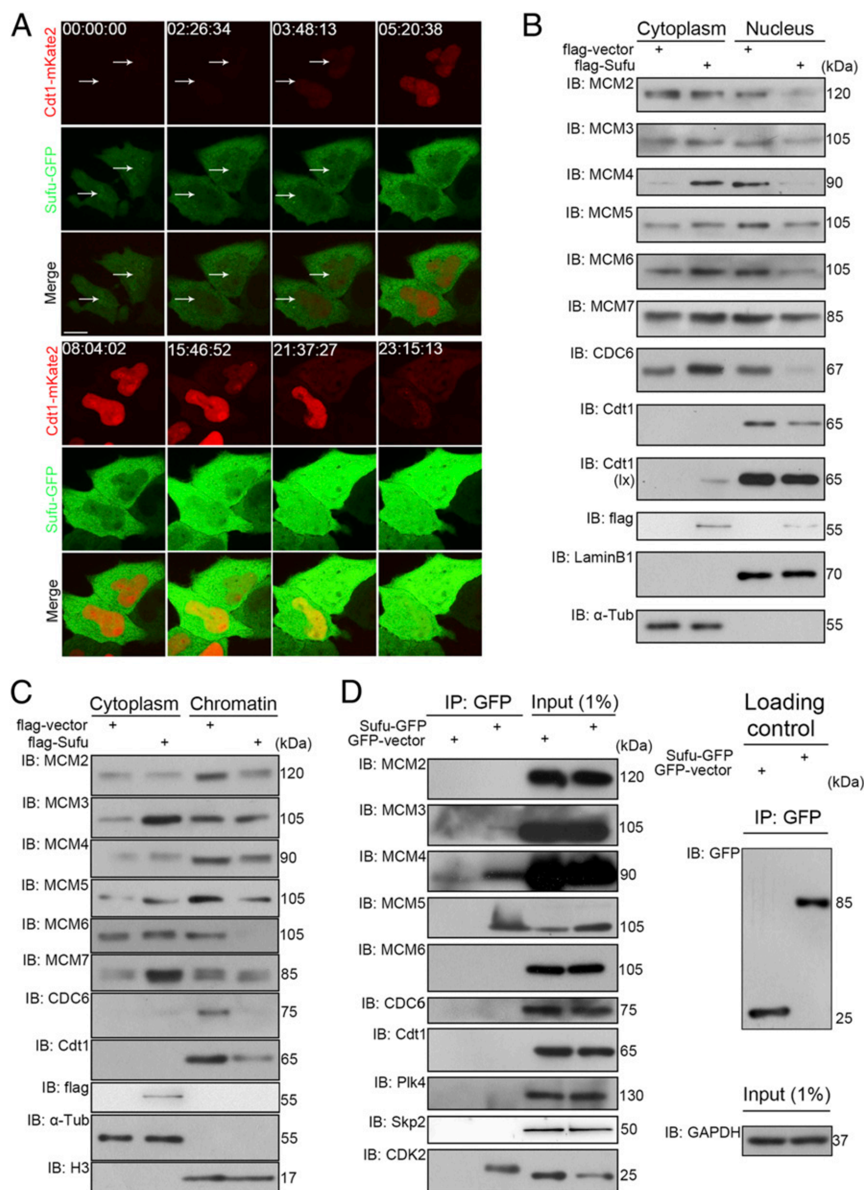

Fig. 5. Sufu inhibits DNA replication licensing via pre-RC component exclusion from the G1 phase nucleus. (*A*) HeLa cells simultaneously expressing Sufu-GFP and Cdt1-mKate2 were synchronized to G1-S transition and released into fresh medium followed by time-lapse microscopy. Note that the intranuclear level of Sufu is higher and intranuclear Cdt 1 is lower than that in the cytoplasm during early G1, and along with nuclear Sufu decreases, Cdt1 accumulates in the nucleus. Time setting, h:min:sec. White arrows, gradually down-regulated nuclear Sufu and low nuclear Cdt1 levels, are shown. (Scale bar, 10 μm.) (*B*) G1 phase HeLa cell lysates expressing flag-Sufu were separated into nuclear and cytoplasmic fractions followed by immunoblotting analysis using specific antibodies for the indicated proteins. Note that excessive Sufu in both the nucleus and cytoplasm resulted in decreases of nuclear CDC6, Cdt1, and MCM2-7. lx, long exposure. (*C*) Immunoblotting analysis of cytoplasmic and chromatin-associated proteins of G1-S transition HeLa cells expressing flag-Sufu. Note that overexpression of flag-Sufu reduced associations of CDC6, Cdt1, and MCM2-7 with chromatin. (*D*) IP and immunoblotting of proteins from Sufu-GFP–expressing HEK 293T cell lysates. Note that CDK2, MCM3, MCM4, and MCM5 were coimmunoprecipitated with Sufu-GFP, whereas Plk4, Cdt1, CDC6, Skp2, and other MCM proteins were not. Three independent experiments for analysis were carried out.

ciliary basal body (mother centriole) to the ciliary tip (34), and both the decrease of Sufu fluorescence intensity around the mother centriole (ciliary base) and the increase of Sufu fluorescence intensity at the ciliary tip suggested that centrosomal Sufu is translocated into the primary cilium once the cell is ready to proliferate no matter the Hh activation (Fig. 7 *A* and *D*). To verify this, we performed time-lapse microscopy on MEFs coexpressing Sufu-GFP and ADP ribosylation factor-like protein 13b (Arl13b)–mKate2, a primary cilium membrane marker, to observe the ciliary translocation of Sufu. We discovered that, while Arl13b-mKate2 distributed evenly throughout the cilium after serum stimulation, Sufu accumulated within the cilium, forming particle-like structures. More interestingly, these ciliary Sufu particles did not go back to the cytoplasm, but instead, they were discharged along with the ciliary scission that occurred just behind the Sufu particles (Fig. 7*E* and Movie S8). These results indicate that the cells actively expel Sufu particles from the cilium into the environment.

Then, we investigated the consequences of the ciliary scission with Sufu particle expulsion. We found that along with ciliary scission under short-time serum stimulation, Sas-6 was efficiently recruited to the centrosomes, and in contrast, the cells, which did

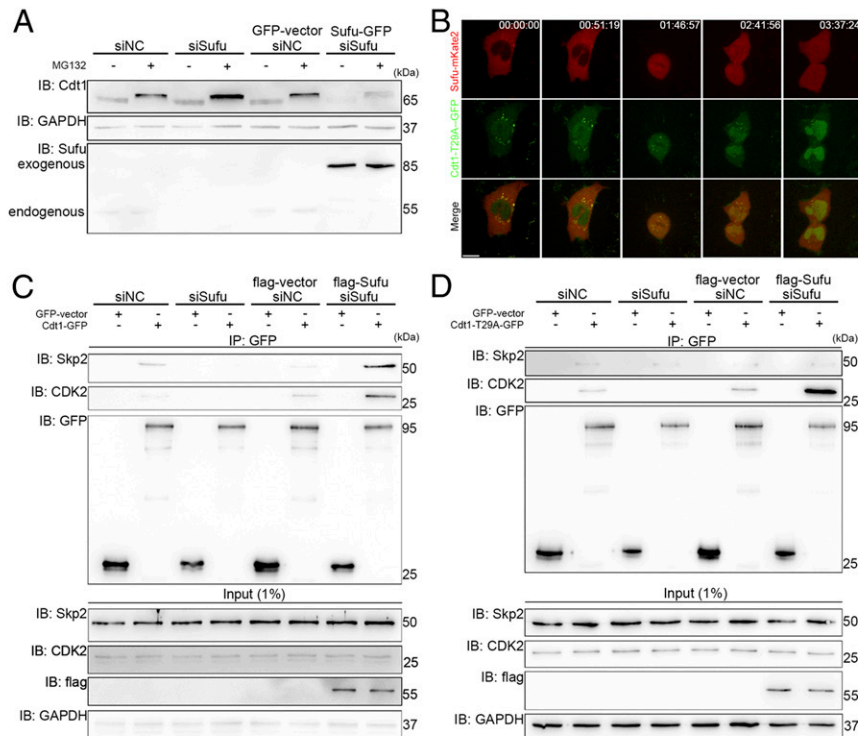

Fig. 6. Sufu promotes intranuclear Cdt1 degradation. (*A*) Cdt1 protein level was measured in HeLa cells with Sufu small interfering RNA (siRNA) knockdown followed by MG132 treatment (left four lanes) or with Sufu-GFP overexpression and Sufu siRNA knockdown followed by MG132 treatment (right four panels). Note that knocking down Sufu increases the amount of Cdt1 (left four panels), whereas expressing Sufu-GFP promoted Cdt1 degradation (right four panels). (*B*) HeLa cells simultaneously expressing Sufu-mKate2 and Cdt1-T29A-GFP were synchronized to G1-S transition and released into fresh medium followed by time-lapse microscopy. Note that although the Sufu level is high in the nucleus during early G1, Cdt1-T29A still accumulates in the nucleus. Time setting, h:min:sec. (Scale bar, 10 μm.) (*C* and *D*) IP and immunoblotting of HEK 293T cell lysates expressing flag-tagged proteins, GFP-tagged Cdt1 proteins, and Sufu siRNA, showing CDK2 and Skp2 interactions. Three independent experiments for each analysis were peformed.

not perform ciliary scission and had a full-length primary cilium, exhibited no Sas-6 centrosomal localization (Fig. 7 *E* and *F*). Interestingly, when SAG was used, Sas-6 was still not able to localize to the centrosome, and if some centrosomal Sufu was translocated into the primary cilium in SAG treatment conditions, more Sufu was translocated from elsewhere to the centrosomal area (Fig. 7 *A* and *C*). These results indicate that activation of the Hh pathway is not sufficient to induce centrosome duplication initiation, consistent with our results that the function of Sufu on centrosome duplication is independent of high Hh activation level. Furthermore, we observed that 1 h serum stimulation was sufficient to down-regulate the phosphorylated CP110 level and triggered translocation of the centrosomal Sufu into the primary cilium (Fig. 7 *A*–*D* and *SI Appendix*, Fig. S7*A*). Moreover, we found that the Sufu aa 250 through 350 mutant interacted with CP110, and the mutant with aa 250 through 350 depletion (Sufu△250–350) abolished this interaction, and more importantly, we observed that Sufu△250–350 overexpression resulted in centrosome amplification (*SI Appendix*, Fig. S7 *B*–*D*). Taken all together, we conclude that the release of suppressive centrosomal Sufu is an essential step for downstream centrosome duplication initiation, and the binding of Sufu with CP110 blocks this release and hence inhibits the centrosome duplication initiation.

Discussion

Both centrosome duplication and DNA replication are tightly coordinated to ensure that only one copy of the daughter centrioles is produced in addition to that DNA replication occurs once and only once per cell cycle (35–38). A number of DNA replication initiation proteins such as ORC1, CDC6, MCM5, and geminin participate in the centrosome duplication initiation (15). In this work, we studied the coupling between initiations of centrosome duplication and DNA replication further and found that Sufu acts as a crucial negative switch. Based on our data, we propose a working model for elucidating the mechanisms of how Sufu as a crucial negative and coordinated switch regulates both initiations of the centrosome and genome duplications (Fig. 7G). In regulating the centrosome duplication initiation, Sufu localizes to the centrosome to facilitate CDK2-mediated phosphorylation of CP110 via binding CP110 and CDK2, which prevents untimely recruitment of Sas-6 for initiation of centrosome duplication. In parallel, Sufu localizes to the nucleus to prevent untimely DNA replication initiation through promoting Cdt1 degradation and inhibiting pre-RC assembly via expelling pre-RC components from the nucleus.

A Functional Sufu Switch Regulates Both Initiations of Centrosome Duplication and DNA Replication. Acting as a negative switch for both centrosome duplication and DNA replication initiations, the amount of Sufu in the nucleus and on the centrosome needs to be carefully regulated and coordinated with the cell cycle control. Although nuclear Sufu has been reported to be involved in Gli nucleocytoplasmic shuttling (39), Sufu is still capable of shuttling into the nucleus on its own in MEFs lacking *Gli* (*Gli1*$^{-/-}$, *Gli2*$^{-/-}$, *Gli3*$^{-/-}$, and *Gli*-null MEFs) (40). It has also been speculated that the accumulation of the Sufu–Gli complex in the nucleus is coupled to their localization at the ciliary tip (40). We also found that PKA phosphorylation on Sufu induces centrosome amplification. Thus, taking our present work with

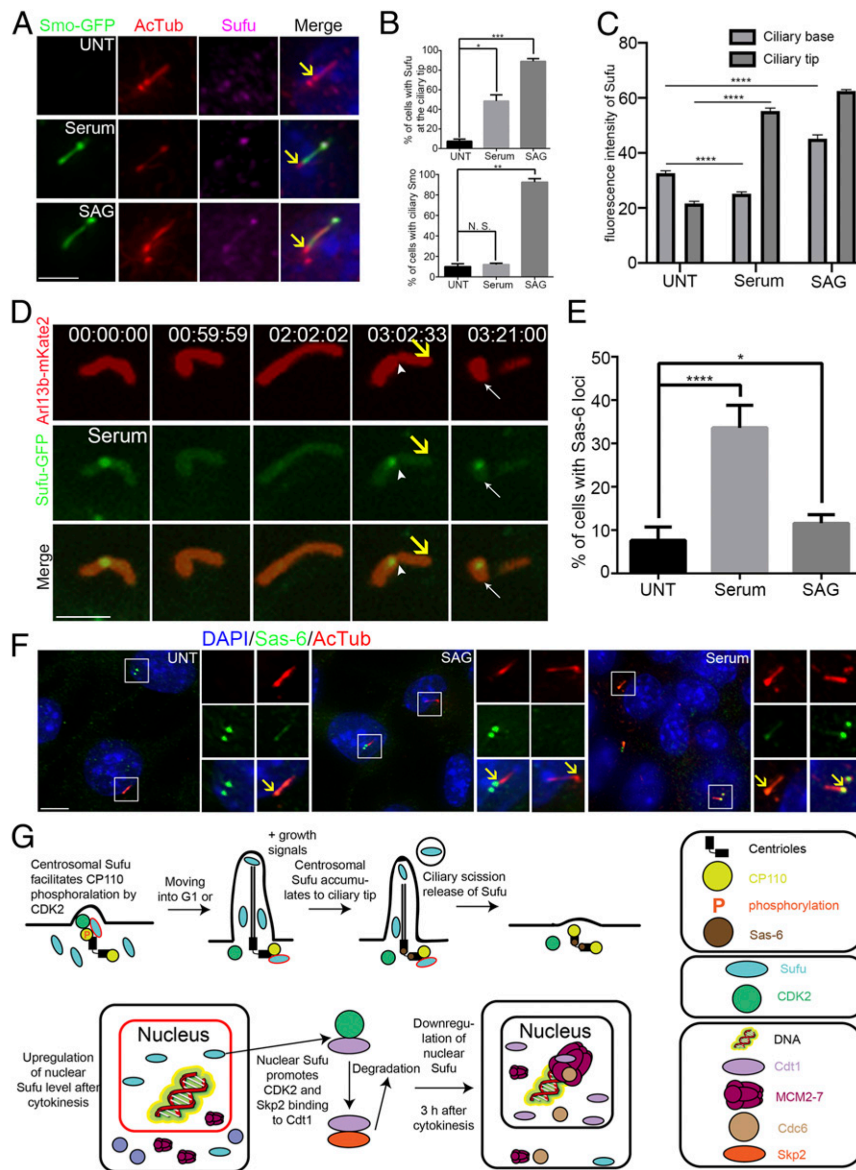

Fig. 7. Inhibitory centrosomal Sufu is eliminated from the ciliary tip along with Hh pathway activation or serum-induced ciliary shedding. (*A*) Immunostaining of stably-Smo-GFP–expressing MEFs (transfected using lentivirus system) with serum or SAG treatment for 20 min (anti-AcTub and -Sufu antibodies). (*B*) Comparisons of percentages of cells with ciliary Smo or with Sufu at the ciliary tip (*A*). (*C*) Fluorescence intensity of Sufu around the ciliary base or at the ciliary tip was measured with ImageJ software. (*D*) Time-lapse images of serum-starved MEFs coexpressing Sufu-GFP and Arl13b-mKate2 with serum stimulation. Only serum, not SAG stimulation, decreased primary cilium length and significantly induced Sas-6 localization to centrosomes. Arl13b marks primary cilium; white arrowheads, vesicle scission points in primary cilium; and white arrow, a vesicle released from the primary cilium. Time setting, h:min:sec. (*E* and *F*) MEFs treated with SAG or serum, immunostained with anti-acetylated AcTub and Sas-6 antibodies (*F*), and comparison of the percentage of cells with Sas-6 foci at the centrosomes at different conditions (*E*). DNA was stained with DAPI (*A* and *F*). (Scale bars, 5 μm.) Yellow arrows (*A*, *D*, and *F*) indicate ciliary base/mature mother centriole area. For means (SD), 100 cells were counted per replicate (*B*, *C*, and *E*), and three independent experiments were carried out for analysis. (*G*) Working model for the roles of Sufu as a negative switch in coordinating both centrosome duplication and DNA replication initiations. During early G1, Sufu localizes to the mother centriole/primary ciliary base of the proliferating cells, where it triggers phosphorylation of CP110 by CDK2 and negatively regulates initiation of centrosome duplication. Before G1-S transition, Sufu is translocated from the mother centriole to the ciliary tip and expelled from the cell via ciliary scission, thereby lifting its inhibitory role on centrosome duplication initiation. Meanwhile, a large fraction of Sufu localizes to the nucleus to induce phosphorylation of Cdt1 by CDK2, promoting binding of Cdt1 with Skp2 for degradation. Intranuclear Sufu also prevents pre-RC components from binding with chromosomes and their nuclear localization. Nuclear Sufu level is down-regulated along with G1 phase progression (3 h after cytokinesis), which enables accumulation of pre-RC components in the nucleus to initiate DNA replication. Thus, Sufu acts as a master negative switch for both initiations of centrosome duplication and DNA replication.

the previously reported together, we proposed that the negative regulations of Sufu on both initiations of centrosome duplication and DNA replication are lifted simultaneously by translocation of the centrosomal Sufu from the mother centriole to the ciliary tip for ciliary scission-regulated release and by the reported degradation of the nuclear Sufu regulated by Fbxl17 (21) and that PKA phosphorylation of Sufu lifts its inhibition on centrosome duplication initiation.

The significance of our findings includes that, first, we identified a function of Sufu, independent of its role in Hh signaling,

to restrain centrosome duplication and DNA replication; second, since Sufu has the dual negative function, we reinforced the association of centrosome duplication with DNA replication; and thirdly and the most importantly, our results enhance the mechanistic understanding of the inhibitory regulation of cells to insure once and only once of both centrosome duplication and DNA replication per cell cycle and may have important implications for further understanding of the mechanisms for precise cell proliferation and cell cycle control.

A Ciliary Vesicle-Expelling Pathway to Quickly Free Negative Regulation. The primary cilium is an antenna-like organelle that projects from the mother centriole to the cell surface. Most mammalian cells have a primary cilium, and with this primary cilium, these cells carry out many signaling pathways, including the Hh pathway, that regulates cell life, proliferation, and differentiation. The primary cilium is dynamic during the cell cycle with disassembly when the mother cell enters mitosis and reassembly after the daughter cells are born. We had previously reported that the majority of the primary cilium in serum starvation–arrested cells at the quiescent phase were dramatically shortened within 2 h after serum stimulation, but the functions of this change were unclear (22). More recently, it was found that primary cilium is shortened through decapitalization (ciliary scission), and the ciliary scission drives quiescence to exit via dynamic remodeling of membrane composition (41). Here, we found that ciliary scission expels inhibitory Sufu, which is translocated from the mother centriole into primary cilium, for initiating centrosome duplication. In this situation, the Sufu around the mother centriole needs to be cast away during duplication initiation. We assume that the ciliary scission pathway may help cells to discharge the unwanted proteins like the inhibitory Sufu by expelling them off the cells.

Crosstalk between primary cilium and nucleus is also a fascinating topic. The similarity between the nuclear pore and the so-called "flagellar pore" at the base of cilia was originally inspired by the shape and size of the two structures (42). Ran, a small GTPase, is a key mediator of nuclear transport (43). Ran and importin-β2 release importin cargo in both the nuclear and ciliary compartments (44). Deletion of ORC1 significantly impairs ciliary assembly without affecting the function of assembled primary cilium and knockdown of ORC1, ORC4, ORC6, CDC6, and Cdt1 blocks translocation of Smo into primary cilium (4). While Cep164, Cep290, NPHP10, and Centrin2 relocalize to the nucleus under conditions of DNA damage (45–48), Cep63 is identified as a substrate of ATM and ATR, activation of which was known to be induced by DNA double-strand breaks, with phosphorylation-induced dispersion from the centrosome (49). In nonciliated cells, the release of negative regulators around the centrosomes may be through translocation from the centrosome to the nucleus for proteolysis in addition to exocytosis.

Up-regulating Sufu Inhibits Tumorigenesis. Germline *Sufu* mutations were firstly described in patients with medulloblastoma (50). Other germline *Sufu* mutations were subsequently found in nevoid basal cell carcinoma syndrome (also known as Gorlin syndrome) (51, 52) and melanoma (53), implicating Sufu as a tumor suppressor. It is known that lifting inhibitory of Sufu will enhance functions of Gli family transcription factors in the nucleus, which promotes downstream cyclin D2 and cyclin E expression and facilitates quiescence exit (16, 54) and excess cytoplasmic Sufu also prevents the nuclear translocation of Gli proteins (40, 55, 56). This work is not only consistent with the previous reports but also presents more direct evidence that knockdown or depletion of Sufu facilitates DNA replication initiation and that over-expression of Sufu represses this process by expelling DNA replication pre-RC components out of the nucleus. With DNA fiber assay, we also found that overexpression of Sufu results in slower replication velocity, suggesting that Sufu has a negative role in DNA replication firing. Post-DNA replication initiation, a small amount of Sufu remaining in the nucleus, is also important to prevent DNA over-duplication.

In addition to the nuclear role for Sufu, the roles of the centrosomal Sufu are also important in preventing tumorigenesis. While over-expression of Sufu represses centrosome duplication initiation, depletion of Sufu causes centrosome overduplication. When introducing Sufu back to the cells with Sufu depletion, it could fully rescue the defects caused by this knockout. Since knocking down or knocking out Sufu results in not only DNA rereplication but also centrosome overduplication, it is obvious that low expression level or disfunction of Sufu may be the causes for some sorts of tumorigenesis.

In summary, we find that Sufu serves as a crucial negative and coordinated switch in regulating initiations of the centrosome and genome duplications. Disfunction of Sufu in our experimental conditions induces centrosome amplification and genome instability. Sufu suppression on tumoriogenesis might be due to preventing untimely initiations and overduplication of both centrosomes and genomes, which directly cause aneuploidy. Our work may have important implications in understanding the mechanisms of cell proliferation, centrosome amplification, genome instability, and tumoriogenesis.

Materials and Methods

Smo-GFP in pCDH vector was cloned from Smo-GFP in pRK5 vector for lentivirus infection to construct stable expression cell line. Flag-Sufu from Sufu-GFP was cloned into the pCMV-Flag-2b vector, and Cdt1 and Arl13b were individually cloned into the pmKate2-N vector. CP110-8A and -8D were mutated from pEGFP-CP110-C1, Cdt1-T29A mutant was mutated from pRK5-Cdt1-GFP, and Sufu- S342A, S342D. S346A, S346D, S342/S346A, and S342/S346D were mutated from pRK5-Sufu-Myc. siSufu: GGACGGCACUUUACAUAUATT (sense: 5′-3′).

Detailed information on cell culture, cell cycle synchronization, cell transfection, β-Gal staining, immunofluorescence, live-cell imaging, λPPase assay, nuclear and cytoplasm fractionation assay, chromatin extraction assay, DNA fiber assay, and antibodies are provided in *SI Appendix, Materials and Methods*.

Data Availability. All study data are included in the article and/or supporting information.

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