Investigation of the interplay between SKP2, CDT1 and Geminin in cancer cells

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Abstract

Geminin has a dual role in the regulation of DNA replication: it inhibits replication factor CDT1 activity during the G2 phase of the cell cycle and promotes its accumulation at the G2/M transition. In this way Geminin prevents DNA re-replication during G2 phase and ensures that DNA replication is efficiently executed in the next S phase. CDT1 was shown to associate with SKP2, the substrate recognition factor of the SCF ubiquitin ligase complex. We investigated the interplay between these three proteins in cancer cell lines. We show that Geminin, CDT1 and SKP2 could possibly form a complex and propose the putative regions of CDT1 and Geminin involved in the binding. We also show that, despite the physical interaction, SKP2 depletion does not substantially affect CDT1 and Geminin protein levels. Moreover, we show that while

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Geminin and CDT1 levels fluctuate, SKP2 levels, differently than in normal cells, are almost steady during the cell cycle of the tested cancer cells.

Introduction

DNA replication takes place in S phase of the cell cycle after the activation of the CDK and CDC7 kinases (Heller et al. 2011; Symeonidou et al. 2012). It is regulated by the formation of the pre-Replicative Complexes (pre-RC) during the preceding gap phase 1 (G1). At the end of DNA replication cells enter a second gap phase (G2) to prepare for mitosis, the phase in which cell division occurs. Several proteins regulate DNA replication. CDT1, in particular, is a DNA replication factor that interacts with CDK and CDC7 kinases (Ballabeni et al. 2009; Chen & Bell 2011; Liu et al. 2004; Nishitani et al. 2004; Sugimoto et al. 2004) and is necessary for pre-RC formation. CDT1 oscillates during the cell cycle to guarantee that DNA is replicated only once before each cell division. It is high in G1, low in S and high again in G2 and mitosis (Ballabeni et al. 2004). The decrease of CDT1 during S phase is key to prevent DNA re-replication and is promoted by the ubiquitin ligase activity associated to Cul4-DDB1-Cdt2 and PCNA activities (Arias & Walter 2006; Guarino et al. 2011; Higa et al. 2006; Hu et al. 2004; Jin et al. 2006; Nishitani et al. 2006; Sansam et al. 2006; Senga et al. 2006; Zhong et al. 2003). The re-accumulation of CDT1 during G2 phase is necessary for preparing for the next round of DNA replication and it depends in part on Geminin, CDK and MAPK activities (Ballabeni et al. 2004; Ballabeni et al. 2011; Ballabeni et al. 2013; Chandrasekaran et al. 2011). Despite the relative stabilization, CDT1 remains a very unstable protein also during G2 phase (Ballabeni et al. 2013); however, the mechanisms regulating this fast turnover are not clear. One hypothesis is that

SKP2, an adaptor protein associated to the SCF ubiquitin ligase whose activity is necessary for cell cycle progression, and previously shown to bind to and ubiquitinate CDT1 (Kondo et al. 2004; Li et al. 2003; Liu et al. 2004; Nishitani et al. 2006; Sugimoto et al. 2004; Takeda et al. 2005), is responsible for CDT1 turnover during G2 phase. Indeed, some studies have suggested that SKP2 could be involved, together with CDK activity, in the regulation of CDT1 levels (Iwahori et al. 2014; Kondo et al. 2004; Nishitani et al. 2006; Tsunematsu et al. 2013), especially in G2 phase or after UV irradiation. Geminin, a binding partner of CDT1 before cell division (McGarry & Kirschner 1998), prevents DNA re-replication in G2 phase by inhibiting CDT1 (Klotz-Noack et al. 2012) and promotes the S phase of the following cell cycle by stabilizing CDT1 (Ballabeni et al. 2004; Ballabeni et al. 2013; Narasimhachar & Coue 2009).

We hypothesized that Geminin, similarly to CDT1, associates with SKP2 whose activity, in turn, regulate the levels of Geminin and CDT1. Since Geminin, CDT1 and SKP2 have been shown to be deregulated in transformed cells and to hold oncogenic potential (Arentson et al. 2002; Chan et al. 2013; Di Bonito et al. 2012; Gstaiger et al. 2001; Hershko 2008; Lin et al. 2009; Montanari et al. 2005; Montanari et al. 2006; Signoretti et al. 2002; Yoshida et al. 2004), elucidating the interplay between these proteins would be important to shed light on the proliferation of tumor cells.

We observed that Geminin associates with SKP2, likely *via* CDT1, and mapped the putative regions of Geminin and CDT1 involved in the binding. Despite the physical interaction, SKP2 levels do not appreciably affect CDT1 and Geminin levels neither during the normal cell cycle nor under conditions of CDT1 instability. These results indicate that, despite its previously reported ability to ubiquitinate CDT1, SKP2 is not the most important factor in the regulation of CDT1 and Geminin proteins levels during G2 phase. Moreover we observed that SKP2 levels,

differently from CDT1 and Geminin levels (Ballabeni et al. 2004; McGarry & Kirschner 1998), are almost stable during the cell cycle of the tested cancer cells. Future studies will be key to elucidate the function of the physical interplay between the three proteins and the significance of the presence of SKP2 protein in the G1 phase of some cancer cell types.

Materials & Methods

Antibodies

The antibodies for the following proteins were used: CDT1 (P26A6 (Ballabeni et al. 2004) or Bethyl A300-786A), Geminin (Santa Cruz 13015), SKP2 (Santa Cruz 7164), FLAG tag (M2 Sigma), HA tag (as previously used (Ballabeni et al. 2004)), Cyclin A (Santa Cruz 596), Cyclin B (Santa Cruz 245), Mcm2 (Santa Cruz 9839), Mcm6 (Santa Cruz 9843), Cdc27 (aka APC3) (Santa Cruz 13154), APC4 (Santa Cruz 21414), CDC7 (as previously used (Ballabeni et al. 2009)), Vinculin (Santa Cruz 25336).

Cell lines and treatments

HeLa, U2OS and 293 cells were incubated in DMEM containing 10% fetal bovine serum at 37 °C with 5% CO_2. Cells were synchronized in S phase by treatment with 2.5 mM thymidine. Cells were synchronized in mitosis by treatment with 50 ng/ml Nocodazole after pre-treatment with thymidine. Cycloheximide (Biomol GR-310) was used 20 mg/ml. MG132 (Biomol PI-102) was used 10 mM.

Plasmids and siRNA

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Plasmids for human CDT1 and human Geminin were previously constructed (Ballabeni et al. 2004; Ballabeni et al. 2009). Plasmids for CDC7 and DBF4 were previously constructed (Ballabeni et al. 2009). S31A mutant of CDT1 was obtained by changing the nucleotide sequence of *CDT1* gene by PCR-base site directed mutagenesis (Stratagene). Plasmid for human SKP2 was a kind gift from Prof. Luca Busino (University of Pennsylvania). siRNA for CDT1 and Geminin were previously used (Ballabeni et al. 2004). siRNA for human SKP2 was from Cell Signaling (mixture of #7753 and #7756). Transient transfections of plasmids were performed by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen), according to the instructions of the manufacturer (for the experiment shown in Fig. 1A, 3A, 3B, S2A and S2B) or by using the Calcium Phosphate transfection method (for the experiment shown in Fig. 1B, 1C, 2A, 4D, S1, S4A, S4B, S4C and S4D). Transfections of siRNA were performed using Oligofectamine (Invitrogen); Opti-MEM Reduced Serum Medium (Invitrogen) was used for the preparation of the siRNA mixtures and then added to the cells in presence of DMEM containing 10% fetal bovine serum.

Immunoprecipitations and western blotting analyses

ANTI-FLAG M2 Affinity Gel from Sigma (#A2220) was used for immunoprecipitations of FLAG-tagged SKP2. Antibodies for HA, Cyclin A, Cyclin B and CDC7 described above were used to immunoprecipitate HA-CDT1, Cyclin A, Cyclin B and CDC7, respectively. Immunoprecipitated and co-immunoprecipitated proteins were detected by western blotting analysis after gel separation, transfer to nitrocellulose membranes and primary hybridizations with the antibodies described above. Secondary hybridizations were performed with HRP-conjugated antibodies for ECL detection or with infrared probe-conjugated antibodies for

detection with Odyssey Infra-red imaging system (Li-Cor). We did not observe differences between the two detection methods with regard to protein detection. Total protein concentrations were usually quantified by Bradford protein assays to load equal amount of protein in each lane of the gels. Equal protein loadings, as well as the quality of the transfer, were also verified by Ponceau staining of the nitrocellulose membranes.

Kinase assays

For *in vitro* kinase reactions the recombinant GST-CDT1 was purified from *E. coli* and used as a substrate. Reactions were completed with 0.1 mM ATP, 5 mCi g-^{32}ATP (Amersham Biosciences) in the following buffer: 50 mM Hepes/KOH (pH 7.6), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 1 mM NaF and protease inhibitors. The reactions were conducted at 30 °C for 30 minutes with moderate shaking and stopped with Laemmli buffer. After separation on 10% polyacrilamide gels and transfer to nitrocellulose membranes, the phosphorylated CDT1 was detected by autoradiography. Recombinant Geminin (either cleaved or not-cleaved from GST) and p53 (cleaved from Maltose Binding Protein moiety) were purified from *E. coli* and used as substrates for kinase assays shown in Fig. S3.

Results

CDT1 and Geminin association with SKP2 in vivo

It was previously shown that CDT1 binds to SKP2 (Kondo et al. 2004; Li et al. 2003; Liu et al. 2004; Nishitani et al. 2006; Sugimoto et al. 2004). We therefore investigated if also Geminin can associate with SKP2. In order to test this hypothesis we expressed recombinant SKP2 and

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Geminin proteins tagged at their N-terminals with FLAG- and Myc-epitopes, respectively. As shown in Fig. 1A exogenous Geminin can associate with SKP2 immunoprecipitate (Fig. 1A). Since under these experimental conditions we were not able to co-immunoprecipitate endogenous Geminin, we decided to increase the expression of exogenous SKP2. To this purpose, we used a HeLa cell line stably expressing FLAG-tagged SKP2 with Myc-tagged Geminin transiently expressed as before. Western blotting analysis showed that also endogenous Geminin could associate with exogenous SKP2 (Fig. 1B). Interestingly, the co-expression of CDT1 led to increased levels of Geminin associated with SKP2 (Fig. 1B). This observation led us to hypothesize that binding between Geminin and SKP2 is mediated by CDT1. To test this hypothesis in vivo, we took advantage of the fact that CDT1 levels drastically decrease in response to UV irradiation (Ballabeni et al. 2009; Kondo et al. 2004). We treated HeLa cells, which previously had been pre-treated or not for 1h30' with the proteasome inhibitor MG132, with UV. 15' after irradiation, we lysed the cells, immunoprecipitated for FLAG and performed western blotting analysis. As expected CDT1 decreased in response to UV, but not when cells were co-treated with MG132 (Fig. 1C). Interestingly, despite there were not major changes in Geminin levels after UV treatment, the fraction associated with FLAG-SKP2 dramatically diminished in cells treated with UV, but not in cells co-treated with MG132 (Fig. 1C). Taken together these results show that not only CDT1, but also Geminin could physically interact with SKP2. These data suggest that the three proteins could possibly associate in the same molecular complex with CDT1 mediating the interaction between Geminin and SKP2 (Fig. 1D).

Characterization of the regions of Geminin involved in the association

To further characterize the interaction between Geminin and SKP2, we tested several deletion mutants of Geminin lacking consecutive sequences of 30 amino acids (Ballabeni et al. 2004). We overexpressed these mutants in 293 cells in combination with FLAG-SKP2. Co-immunoprecipitation experiments showed that mutants lacking amino acids 91-120 and 121-150 were not able to interact with SKP2 (Fig. 2A and 2B). We also noticed that endogenous Geminin was not co-immunoprecipitated when the exogenous versions of Geminin competent in SKP2 binding were co-expressed (Fig. 2A), suggesting that ectopically expressed Geminin prevents endogenous Geminin binding by competing for the same binding site. Since amino acids 91-120 were previously shown to be involved in the binding to CDT1 (Ballabeni et al. 2004), these results confirm that Geminin could possibly bind to SKP2 through its association with CDT1.

Characterization of the regions of CDT1 involved in the association

We then further characterized the interaction between CDT1 and SKP2. To this purpose, we tested a version of CDT1 with Serine 31 mutated to Alanine. This Serine is a putative CDK phosphorylation site that has recently been shown to be phosphorylated *in vivo* (Miotto & Struhl 2011). Since this residue is close to Threonine 29, a residue involved in SKP2 binding (Takeda et al. 2005), we hypothesized that phosphorylation of Serine 31 regulates the interaction between CDT1 and SKP2. To test this, we transiently overexpressed FLAG-tagged SKP2, HA-tagged CDT1 (either wild-type or mutated on Serine 31) and Myc-tagged Geminin. As shown in Fig. 3A and 3B, wild-type CDT1 co-immunoprecipitates SKP2, while the Serine 31 mutant does not (Fig. 3A and 3B). Remarkably, Geminin overexpression did not affect the binding of SKP2 to CDT1 (Figure 3B), suggesting that binding of CDT1 to SKP2 does not depend on Geminin.

Because Serine 31 is located in a SPAR sequence (a putative CDK phosphorylation site) and CDT1 was previously shown to be phopshorylated by CDK (Chen & Bell 2011; Liu et al. 2004; Sugimoto et al. 2004), we tested if Serine 31 mutation affects the phosphorylation by CDK. To this purpose, we performed kinase assays using immunoprecipitation of Cyclin A and Cyclin B in lysates of HeLa cells. Recombinant *E. coli*-purified GST-tagged CDT1, either wild-type (WT) or mutated on Serine 31, was used as a substrate. We observed that the WT version was phoshorylated very efficiently while the mutated version was phosphorylated much less efficiently by cyclin A and cyclin B-associated kinase activities (Fig. 3C and 3D). Because CDC7 kinase was also reported to be able to bind to and phosphorylate CDT1 (Ballabeni et al. 2009) (and Fig. S1), we performed a similar kinase assay after immunoprecipitation of CDC7 kinase overexpressed in HeLa cells. Again, we observed that the mutant was phosphorylated much less efficiently (Fig. 3E). These results suggest that Serine 31 of CDT1, located in the N-terminal regulatory region, could be an *in vivo* phosphorylation site for CDK and CDC7 kinases. Moreover, these results show that Serine 31, similarly to Threonine 29, is likely involved in the regulation of the association between CDT1 and SKP2 (Fig. 3F).

SKP2 levels do not substantially affect CDT1 and Geminin levels

It was previously reported that SKP2 protein binds to and ubiquitinates CDT1 (Kondo et al. 2004; Li et al. 2003; Liu et al. 2004; Nishitani et al. 2006; Sugimoto et al. 2004). Moreover, it was suggested that SKP2 regulates CDT1 levels during G2 phase (Nishitani et al. 2006). Therefore, we tested if SKP2 affects CDT1 protein levels in G2 phase and mitosis. To this purpose, we used siRNA to decrease the levels of SKP2. We synchronized U2OS cells before treating them with siRNA for SKP2 and collecting them in the G2 phase (Fig. 4A). Cells were

grown with and without the protein synthesis inhibitor cycloheximide for 1 hour before harvesting. Despite the strong decrease in SKP2 levels, no substantial change in CDT1 protein levels was observed, either in presence or absence of cycloheximide (Fig. 4B). We also tested if a decrease in SKP2 levels can change CDT1 levels in G2 and mitosis after experimental depletion of Geminin, a condition that leads to decrease of CDT1 levels (Ballabeni et al. 2004; Ballabeni et al. 2013). To this purpose, we treated U2OS cells with siRNA for SKP2 and/or Geminin and, after synchronization, collected them at the end of the G2 phase or mitosis (Fig. S2A). We observed that depletion of SKP2 did not affect the levels of CDT1 protein in G2 phase and mitosis, either with normal or decreased levels of Geminin protein (Fig. S2A). To further corroborate these results, we repeated the experiment but this time we decreased the duration of the siRNA treatment to prevent any possible small alteration of the cell cycle structure. We therefore synchronized and treated U2OS cells with siRNA at the S/G2 transition by using a previously developed protocol (Ballabeni et al. 2004) (Fig. 4C) and we then collected the cells arrested in mitosis. We observed again that depletion of SKP2 protein did not affect the levels of CDT1 protein either under normal or decreased levels of Geminin protein (Fig. 4D). Geminin levels, also, were not altered by the depletion of SKP2 (Fig. 4B, 4D and S2A). Finally, we tested if SKP2 depletion prevents CDT1 decrease after UV irradiation. To this purpose, we treated U2OS cells with siRNA for SKP2, synchronized them in G2 phase and treated them with a UV dose of 30 J/m^2 for 45 minutes. We observed that depletion of SKP2 protein did not affect the levels of CDT1 after UV irradiation (Fig. S2B). We also observed that SKP2, similarly to CDT1, is significantly decreased after UV irradiation (Fig. S2B). In addition we noticed that SKP2 levels, similarly to CDT1 levels, are decreased when Geminin levels are decreased (Fig. 4D and S2A); however, differently from CDT1, the decrease of SKP2 levels seems to be due to an

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indirect effect of the siRNA transfection (Fig. S3). Overall these results indicate that, despite SKP2 protein likely physically interacts with CDT1 and Geminin proteins, it is not the most important factor in the regulation of their levels, under either physiological or non-physiological conditions.

SKP2, CDT1 and Geminin levels are simultaneously high only in G2 and mitosis

CDT1 and Geminin were previously shown to be present in G2-M-G1 and S-G2-M phases, respectively (Ballabeni et al. 2004; McGarry & Kirschner 1998; Nishitani et al. 2004; Nishitani et al. 2000). Here we tested the levels of SKP2 protein in G1 and G2-M phases. First, HeLa cells were synchronized in mitosis and released in G1 phase for several hours (Fig. 5A). We observed that, while Geminin, as expected, was almost undetectable during G1, SKP2 protein levels were relatively abundant (Fig. 5A). We also checked the levels of SKP2 on chromatin. We noticed that whereas CDT1 was, as expected, present in the chromatin-containing fraction after mitotic exit (Ballabeni et al. 2004), SKP2 protein was undetectable. Therefore, despite SKP2 protein was previously shown to be a target of APC (Bashir et al. 2004; Wei et al. 2004), our results show that in the tested cancer cells SKP2 is present during G1 phase and its levels fluctuate much less than Geminin levels during the M-G1 transition. Then, to test the levels of SKP2 protein in G2 and mitosis (the only phases in which both CDT1 and Geminin are present), we synchronized HeLa cells in these two phases. We noticed that all three proteins are present during G2 and mitosis (Fig. 5B). In parallel, we also treated the cells for 1 hour with the proteasome inhibitor MG132. We observed that only CDT1 protein levels were significantly affected by the inhibition of the proteasome (Fig. 5B), indicating that SKP2 and Geminin turnover is slow during G2 and mitosis. Moreover, we also released the cells in G1 phase for 1 hour. Again, we noticed that

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whereas Geminin levels significantly decreased, SKP2 levels did not (Fig. 5B). We also noticed that the addition of the proteasome inhibitor MG132 during G1 altered the levels of CDT1 and Geminin much more than the levels of SKP2 (Fig. 5B), indicating that the turnover of SKP2 during G1 is much slower. To confirm that SKP2 protein is present in G1 and has a slow turnover, we also tested another cell line. To this purpose, we synchronized U2OS cells in mitosis and released them for 3 hours before treating them with and without the proteasome inhibitor MG132 for 1 hour. In agreement with the previous results, we noticed that SKP2 protein is present in G1 and has a slow turnover compared to CDT1 and Geminin (Fig. 5C). These results confirm the previous observations about the fluctuations of CDT1 and Geminin and show that SKP2, whose levels were reported to be high in S phase (Bashir et al. 2004; Lisztwan et al. 1998; Wei et al. 2004), is in fact present in all phases of the cell cycle and only mildly fluctuating in the tested cancer cells (Fig. 5D).

Discussion and Conclusions

SKP2 was previously shown to bind to and ubiquitinate CDT1 (Kondo et al. 2004; Li et al. 2003; Liu et al. 2004; Nishitani et al. 2006; Sugimoto et al. 2004; Takeda et al. 2005), and to regulate its levels in G2 phase (Nishitani et al. 2006; Tsunematsu et al. 2013). The recent observation that CDT1 turnover is very high (despite the high protein levels) in G2 phase (Ballabeni et al. 2013) led us to hypothesize that SKP2 indeed might have a role in regulating CDT1 levels in G2 and mitosis. On the contrary, we have observed that SKP2 depletion does not substantially affect CDT1 levels in G2 phase and mitosis, even when CDT1 levels are experimentally decreased either by Geminin depletion or UV irradiation. Given that SKP2 protein is not completely depleted from the cells after siRNA treatment, we cannot totally rule out the possibility that only

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minimal levels of SKP2 are sufficient to regulate the levels of CDT1. However, considering that the depletion of SKP2 was very effective in our experimental conditions, we can postulate that SKP2 is most likely not a major regulator of CDT1 turnover during G2 and mitosis. It is possible that multiple ubiquitin ligases regulate CDT1 turnover during G2 and mitosis and that only the simultaneous inhibition of all these activities would affect CDT1 levels.

In this report we have also shown that SKP2, CDT1 and Geminin, as previously proposed (Takeda et al. 2005), might form a complex inside the cells. Our data suggest that CDT1 might function as a bridge between SKP2 and Geminin. We have also shown that the mutation of Serine 31 in a SP motif, which affects the phosphorylation by CDK and CDC7 kinases *in vitro,* is probably involved in the association with SKP2 *in vivo* (this is different from what proposed in a previous report (Takeda et al. 2005) and we think that the discrepancy is plausibly due to the slightly different experimental conditions). Whereas the SP motif was already known to be a phosphorylation site for CDK kinases, the same was not reported for CDC7 kinase. Indeed we observed that CDC7 kinase is able to *in vitro* phosphorylate also other recombinant proteins on SP motifs, including Geminin and p53 (Fig. S4 and data not shown); these results suggest that this motif could possibly be a phosphorylation site *in vivo*.

Moreover, our results show that SKP2 is present throughout the cell cycle in the tested cancer cell cells. Thus, while the interaction between the three proteins likely occur only in G2 phase and mitosis, CDT1 and SKP2 could perhaps interact also in G1 phase in these cancer cells (Fig. 5D).

Future studies will have to shed light on the functional role of the putative SKP2-CDT1-Geminin physical interaction and to elucidate the ubiquitin ligases involved in the rapid turnover of CDT1 protein during G2 phase. Future investigations will also be important to clarify the significance

of SKP2 protein presence during the G1 phase of some cancer cell types. This information will be useful to advance our understanding of the molecular mechanisms regulating cell cycle and cell transformation.

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Figure legends

Figure 1. Geminin association with CDT1-SKP2 (A) Geminin associates with SKP2 *in vivo*. Myc-tagged Geminin and/or FLAG-tagged SKP2 expression plasmids were transfected in HeLa cells for 24 hours. Cell lysates were prepared and SKP2 was immunoprecipitated with FLAG antibody. Western blotting analyses for the indicated total or immunoprecipitated proteins are shown. Under these conditions only exogenous Geminin was detected associated to SKP2. **(B)** Geminin and CDT1 associate with SKP2 in vivo. Myc-Geminin and HA-CDT1 plasmids were transfected as indicated for 48 hours in HeLa cells stably overexpressing FLAG-SKP2 protein. SKP2 was immunoprecipitated with the FLAG antibody and Western blotting was performed for the indicated total or immunoprecipitated proteins. Under these conditions also endogenous

Geminin was detected associated to SKP2. **(C)** Geminin and CDT1 do not bind to SKP2 after UV irradiation. HeLa cells stably overexpressing SKP2 were treated with a dose of 30 J/m^2 of UV irradiation for 15 minutes. Cells were optionally treated with the proteasome inhibitor MG132 for 90 minutes before UV irradiation. Cells were lysed 15 minutes after UV-irradiation, and FLAG-tagged SKP2 was immunoprecipitated with the FLAG antibody. Western blotting analyses for the indicated total or immunoprecpitated proteins are shown. **(D)** Schematic working model of the interaction between CDT1, Geminin and SKP2, before or after UV irradiation.

Figure 2. Mapping of the regions of Geminin involved in the association (A) Mapping of the amino acid residues in Geminin required for the interaction with SKP2. FLAG-SKP2 and deletion mutants of HA-tagged Geminin were transfected in 293 cells and immunoprecipitations for FLAG were performed. Western blotting analyses for the indicated total or immunoprecpitated proteins are shown. (B) Schematic representation of the portions of Geminin protein (residues 91-150) required for the association with SKP2.

Figure 3. Mapping of the regions of CDT1 involved in the association (A) HA-CDT1, Myc-Geminin and FLAG-SKP2 were transfected in HeLa cells as indicated. A version of HA-CDT1 with Serine 31 substituted by Alanine was also tested. Western blotting analyses for the indicated proteins are shown. Mcm2, Mcm6 and Cyclin A were used as controls for loading. **(B)** CDT1 mutated on Serine 31 does not interact with SKP2. Cell lysates of transfected HeLa cells shown in (A) were used for immunoprecipations using the HA antibody. Western blotting analyses for the indicated immunoprecipitated proteins are shown. **(C)** Cyclin A2 associated kinase activity

phosphorylates CDT1 WT more efficiently than the mutant S31A. Cyclin A was immunoprecipitated from HeLa cells and used for kinase assays with GST-CDT1 (WT or S31A) as substrates. Autoradiographies for ^{32}P are shown. **(D)** Cyclin B1 associated kinase activity phosphorylates CDT1 WT more efficiently than the mutant S31A. Cyclin B was immunoprecipitated from HeLa cells and used for kinase assays with GST-CDT1 (WT or S31A) as substrates. Autoradiographies for ^{32}P are shown. **(E)** CDC7 phosphorylates CDT1 WT more efficiently than the mutant S31A. Overexpressed CDC7 was immunoprecipitated from HeLa cells and used for kinase assays with GST-CDT1 (WT or S31A) as substrates. Autoradiographies for ^{32}P are shown. **(F)** Schematic representation of domains of human CDT1 protein. Threonine 29 and Serine 31 seem to be both involved in the binding to SKP2. D-box represents the destruction boxes targeted by Cdh1-APC. Cy motif represents the Cyclin binding motif. PIP domain is the target of PCNA associated Cdt2-Cul4-DDB1. Domains involved in the binding to Geminin, MCM and CDC7 are also shown.

Figure 4. Decreased levels of SKP2 protein do not substantially affect CDT1 and Geminin protein levels (A) Schematic experimental outline for results shown in panel B. **(B)** Decreased SKP2 levels do not affect CDT1 and Geminin levels. U2OS cells were first synchronized in mitosis, then released for 7 hours and transfected with siRNA targeting SKP2 or mock. Thymidine was then added to arrest cells in S phase for 16 hours and released for 10 hours to have them in the G2 phase. Cells were then optionally treated with cycloheximide for 1 hour. Western blotting analysis is shown for the indicated proteins. Mcm2 was used as a loading control. **(C)** Schematic experimental outline for results shown in panel D. **(D)** Decreased SKP2 levels do not affect CDT1 levels even after interference for Geminin. U2OS cells were treated

with thymidine and released for 5 hours. Cells were then transfected as previously reported (Ballabeni et al. 2004) with siRNA for CDT1 or Geminin and/or SKP2. Nocodazole was added 4 hours after transfections and cells were incubated for other 16 hours before harvesting of the shake-off cells (mitotic cells). Western blotting analysis is shown for the indicated proteins. Mcm2 protein was used as a loading control.

Figure 5. Levels of SKP2, CDT1 and Geminin proteins during the cell cycle of HeLa and U2OS cancer cells (A) HeLa cells were synchronized in mitosis and released for the indicated times. Western blotting analysis for the indicated proteins is shown. CDT1 and SKP2 levels were also evaluated in the chromatin containing fraction, isolated as previously reported (Ballabeni et al. 2009). **(B)** HeLa cells were synchronized at the end of the G2 phase or mitosis as previously reported (Ballabeni et al. 2004). Two samples of cells were also released from mitosis in G1 phase for 3 hours. Cells in G2, mitosis or G1 phases were alternatively treated with the proteasome inhibitor MG132 for 1 hour. Western blotting analysis for the indicated proteins is shown. CDT1 migrates slower in SDS-PAGE in G2 phase and mitosis due to phosphorylation (Ballabeni et al. 2004). **(C)** U2OS cells were synchronized in mitosis and released in G1 phase for 3 hours. In parallel cells were also treated with the proteasome inhibitor MG132 for 1 hour. Western blotting analysis for the indicated proteins is shown. **(D)** Working model depicting the timing of SKP2-CDT1-Geminin interactions during the cell cycle of certain cancer cell types.

Figure S1. CDC7 kinase phosphorylates CDT1 *in vitro*. U2OS cells were transfected with plasmids expressing CDC7 kinase (tagged with HA) and its activator DBF4 (tagged with Myc) or not transfected. After cell lysis, immunoprecipitations for CDC7 (or with a control unrelated

antibody) were performed and the immunoprecipitates were used for kinase assays with recombinant GST-CDT1 as a substrate. A mutant of CDC7 with an amino acid substitution (D to N) at residue 196 in the ATP hydrolysis site was used as a negative control (Ballabeni et al. 2009). Autoradiography for ^{32}P is shown.

Figure S2. Reduction in SKP2 protein levels does not affect CDT1 and Geminin protein levels (A) Reduction in SKP2 levels does not affect CDT1 and Geminin levels. U2OS cells were transfected with control siRNA or siRNA for Geminin and/or SKP2. The cells were incubated for 10 hours and then treated with thymidine for 20 hours to synchronize them in S phase. The cells were then released in the presence of Nocodazole and incubated for other 16 hours before harvesting of the adherent cells (G2 cells) or the shake-off cells (mitotic cells), as previously reported (Ballabeni et al. 2004). Western blotting analysis is shown for the indicated proteins. APC4 was used as a loading control. CDC27 different mobility is shown as a proof of good separation between G2 and mitotic cells (Ballabeni et al. 2011). **(B)** Reduction in SKP2 levels does not affect CDT1 levels after UV irradiation. U2OS cells were treated with siRNA for SKP2 or control and, 12 hours later, thymidine was added to synchronize the cells in S phase. After release from thymidine, cells were incubated in Nocodazole for 14 hours until 20% of the cells were mitotic and in suspension (no differences were observed between control and SKP2 siRNA-treated cells, indicating no significant alterations of the cell cycle). Attached cells (G2 phase cells) were at this point irradiated with a dose of 30 J/m^2 of UV and incubated for additional 45 minutes. After cell lysis, western blotting analyses were conducted for the indicated proteins. APC4 and Cyclin A were used as control for loading and synchronization in G2 phase, respectively.

Figure S3. SKP2 protein levels decrease after Geminin depletion is due to indirect effects of siRNA treatment (A) Normal U2OS cells or U2OS cells expressing WT Geminin or Geminin mutated in the binding region (amino acids 112-118) for CDT1 (Ballabeni et al. 2004) were treated in S/G2 phase with siRNA for Geminin, as previously described (Ballabeni et al. 2004). Cells were then synchronized at the end of G2 as previously reported (Ballabeni et al. 2004). Western blotting analysis for the indicated proteins is shown. Vinculin is used as a loading control. **(B)** mRNA for SKP2 was quantified in U2OS cells treated as in (A) by quantitative RT-PCR. Standard deviations are shown. Samples were normalized to b-actin.

Figure S4. CDC7 kinase phosphorylates Geminin and p53 on SP motifs *in vitro* (A) CDC7 phosphorylates Geminin *in vitro*. HeLa cells were transfected with plasmids expressing CDC7 (or negative control mutant D196N) and activating partner DBF4. Immunoprecipitations for CDC7 were then used to perform kinase assays with the recombinant GST-Geminin as a substrate. Autoradiography for ^{32}P is shown. **(B)** CDC7 *in vitro* phosphorylates Geminin on Serine 64, part of a SP motif. HeLa cells were transfected with plasmids expressing CDC7 (or negative control mutant D196N) and activating partner DBF4. Immunoprecipitations of CDC7 were then used to perform kinase assays with the recombinant GST-Geminin WT or S64A (Serine 64 substituted with an Alanine) as a substrate. Autoradiography for ^{32}P is shown. **(C)** Geminin competes with CDT1 for phosphorylation by CDC7 kinase *in vitro*. Kinases assays were performed with CDC7 and DBF4 proteins purified from baculovirus infected insect cells. CDT1 was also purified from baculovirus infected cells. Geminin was produced as GST-tagged fusion protein in *E. coli* (see (A) and (B)) and the GST moiety was cleaved by thrombin

digestion. In vitro kinase assays were performed with CDC7, DBF4, CDT1 and, optionally, Geminin. Autoradiography for ^{32}P is shown. **(D)** CDC7 phosphorylates p53 *in vitro* on Serine 46, part of a SP motif. U2OS cells were transfected with plasmids expressing CDC7 and DBF4 or mock transfected. After cells lysis, immunoprecipitations of CDC7 (or control) were performed. Kinase assays were performed using p53 as a substrate. p53 was purified from *E. coli* as a MPB (maltose binding protein) fusion protein and the MBP moiety was cleaved before the kinase assay. Two mutants of p53 (with substitutions either in Serine 46 or Threonine 55) were used. These two mutants were selected after a screening with deletion mutants of p53 to map the phosphorylation domain (data not shown). Autoradiography for ^{32}P is shown.

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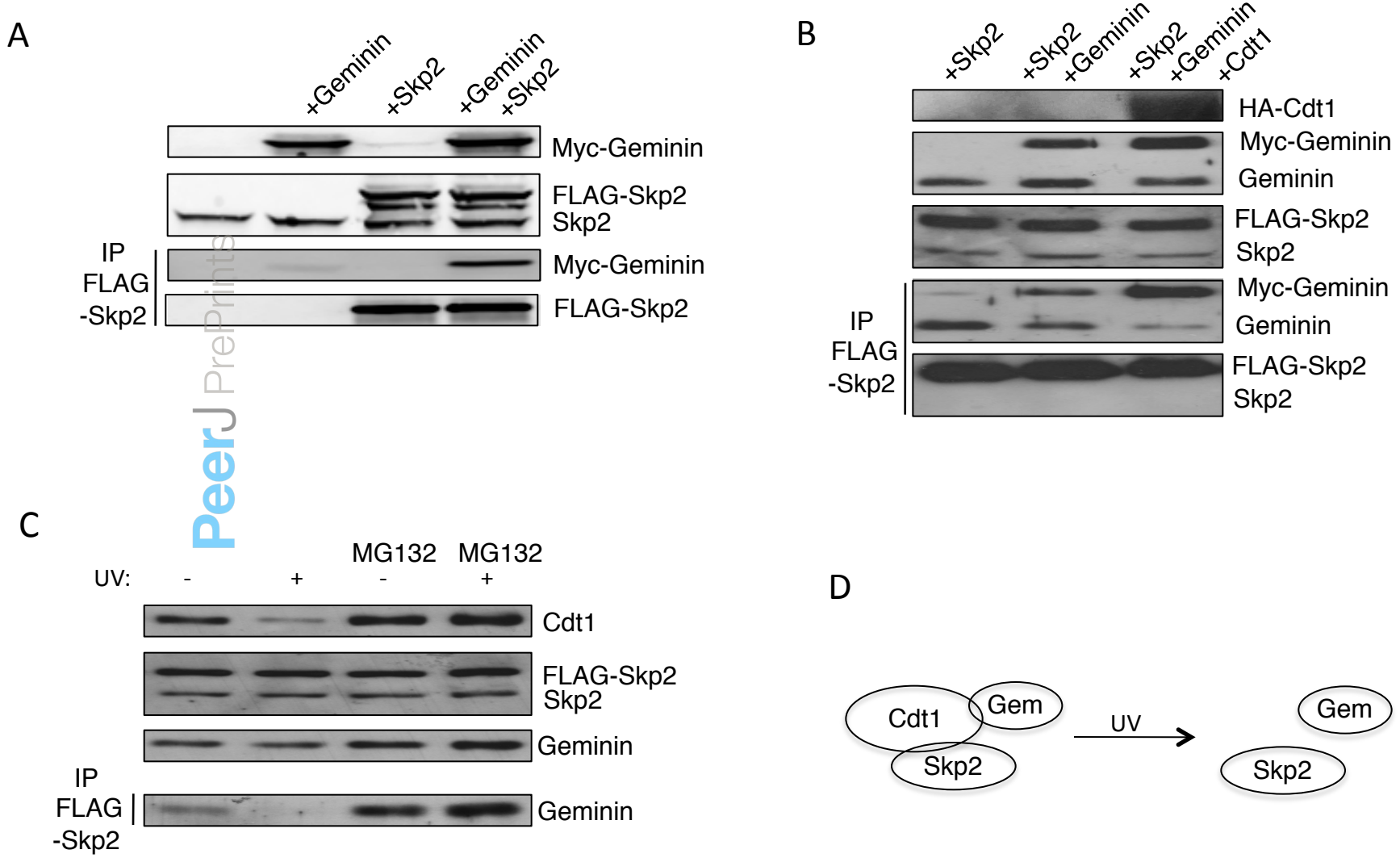
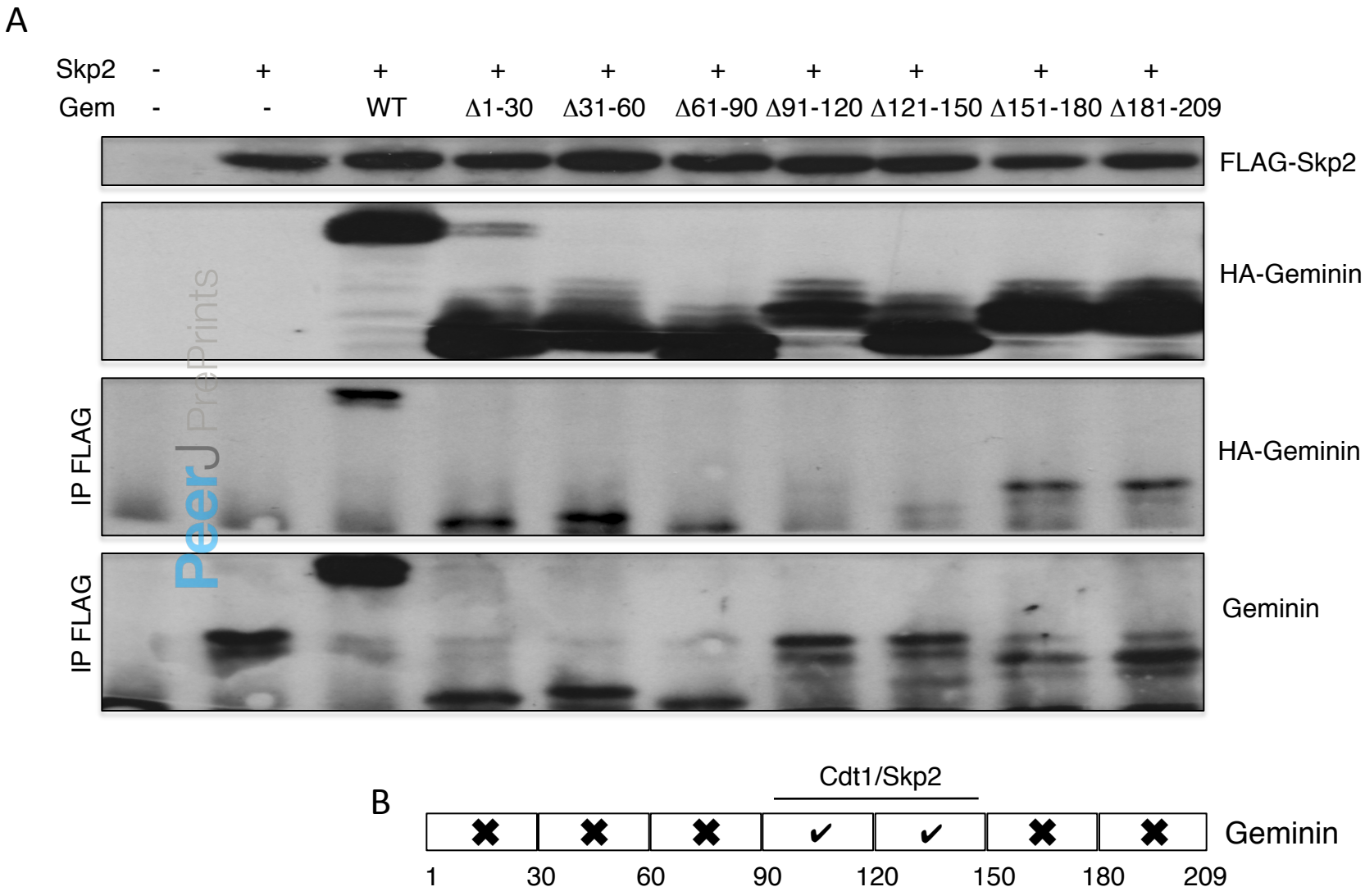


Figure 1



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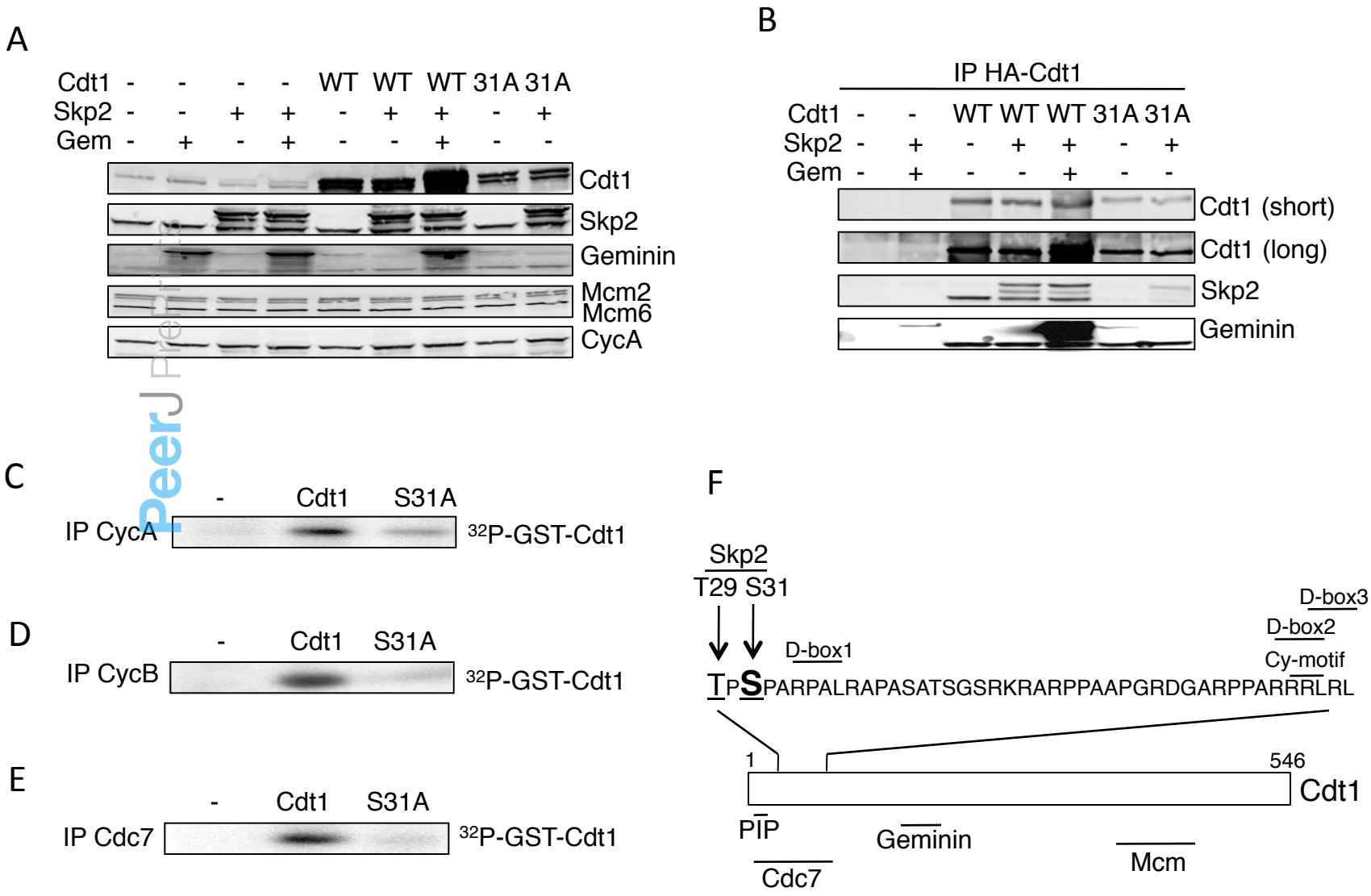

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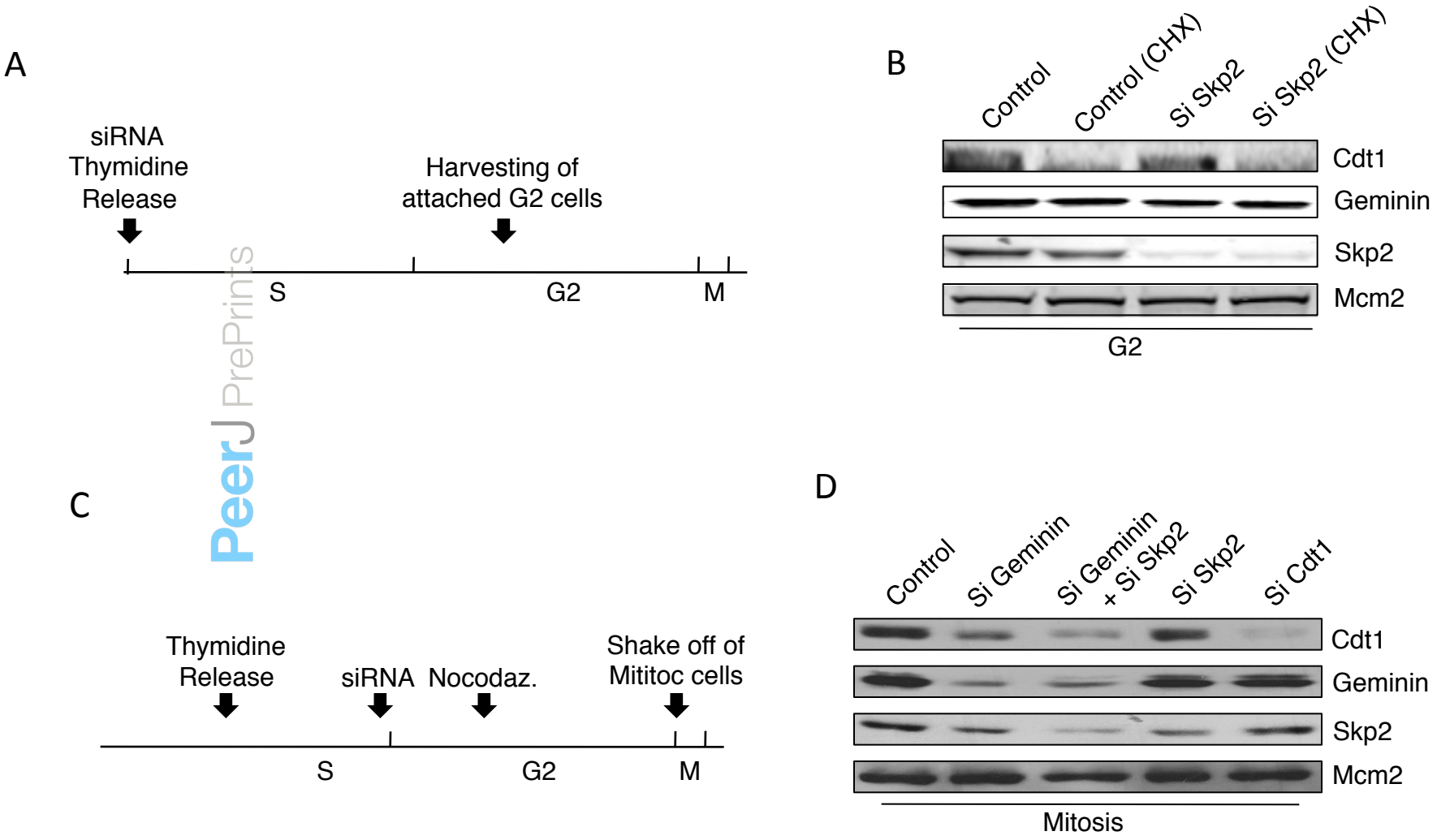

Figure 4

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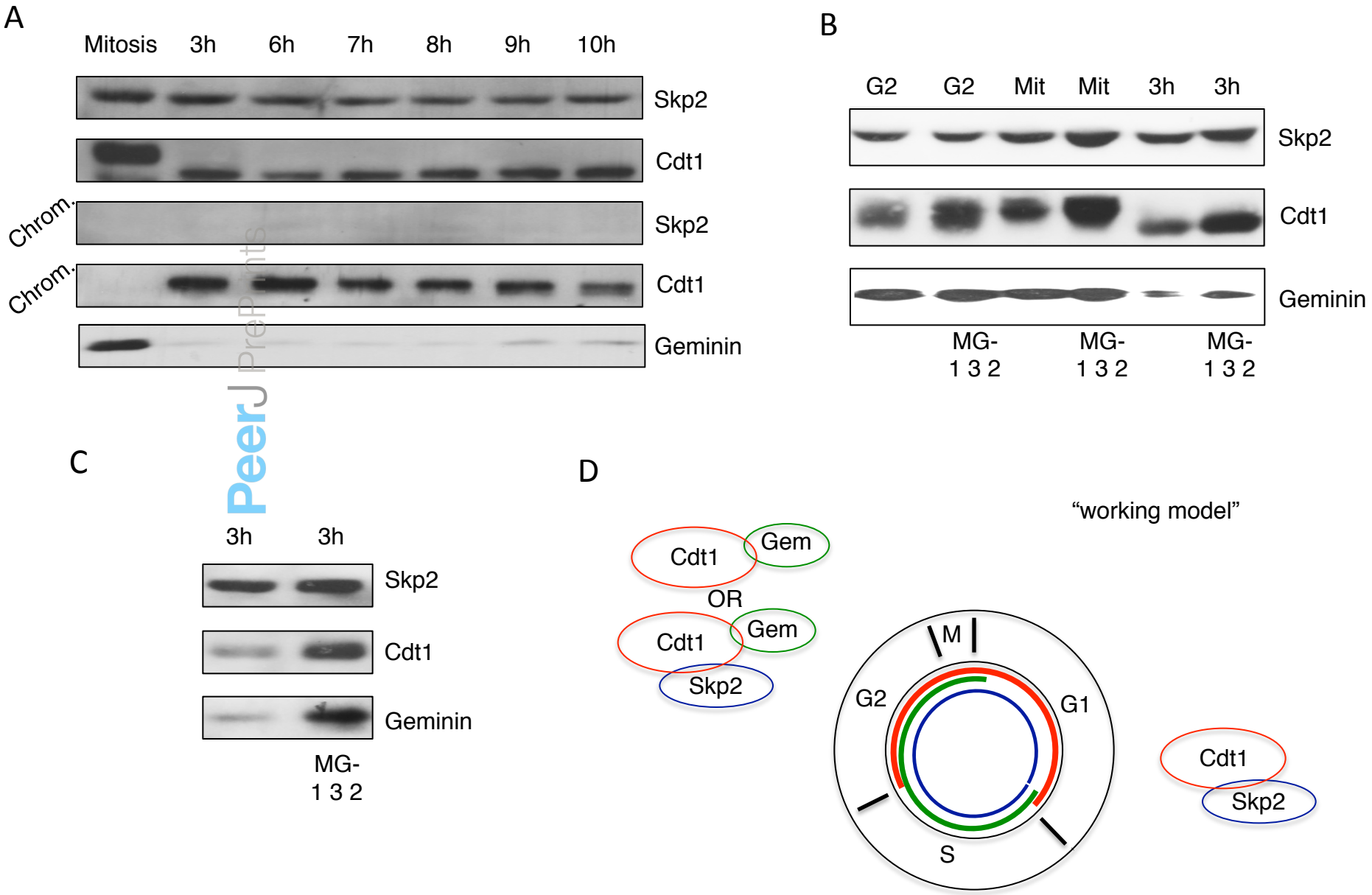

Figure 5

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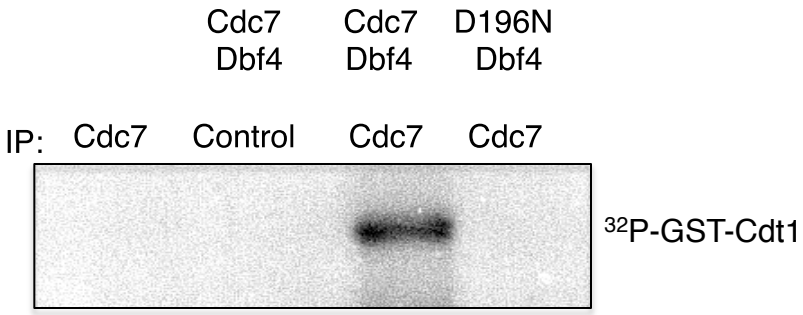

Figure S1

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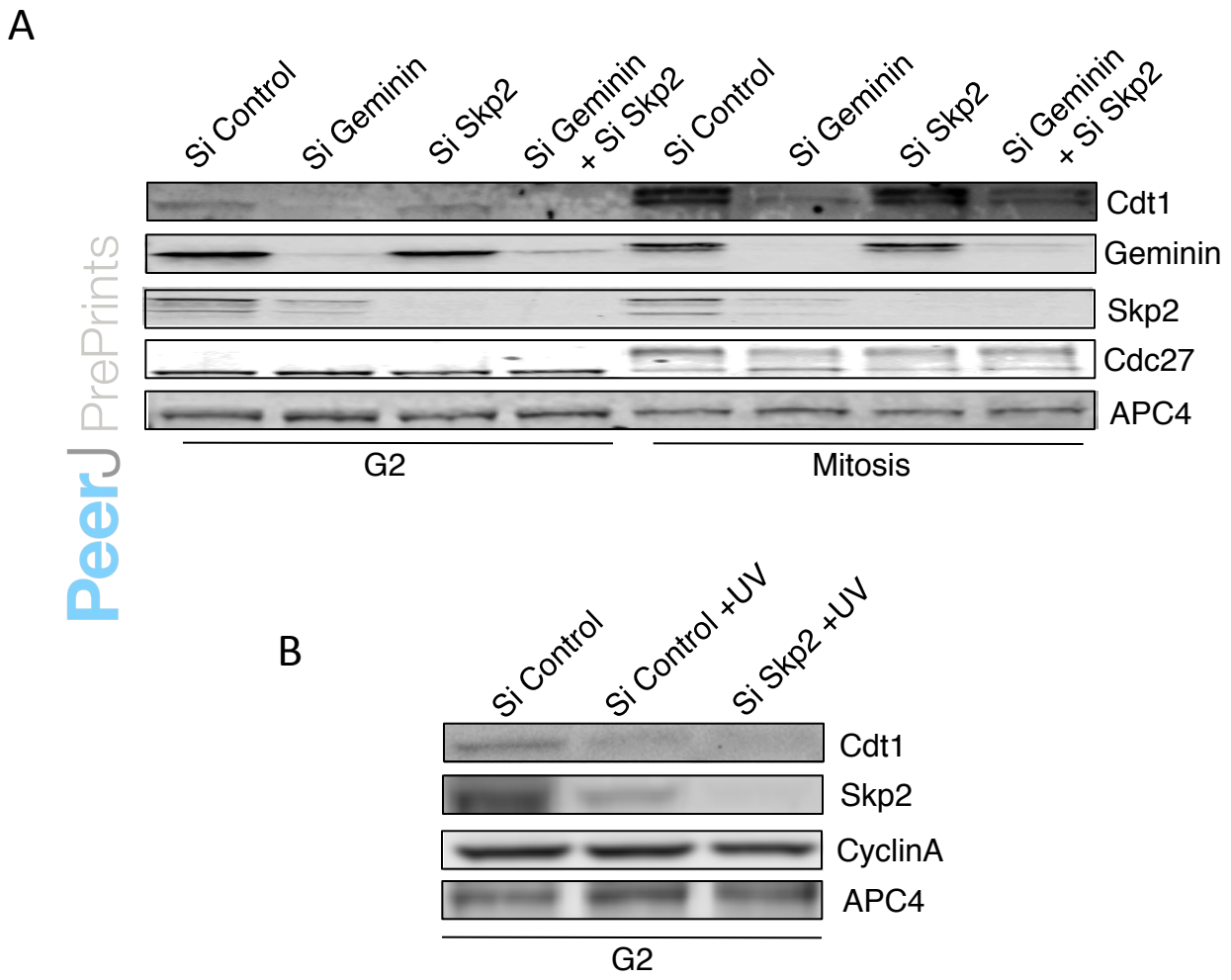

Figure S2

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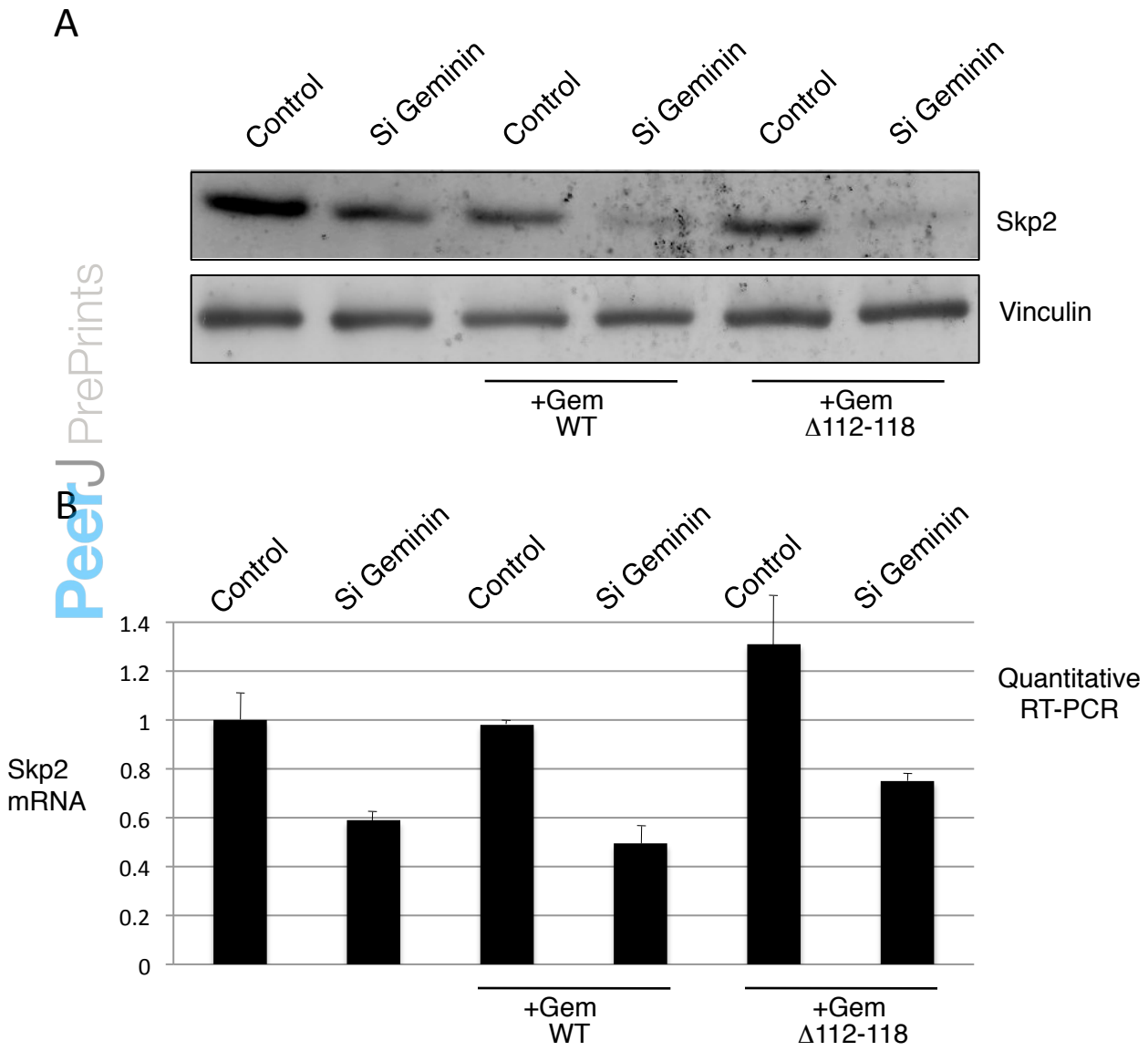

Figure S3

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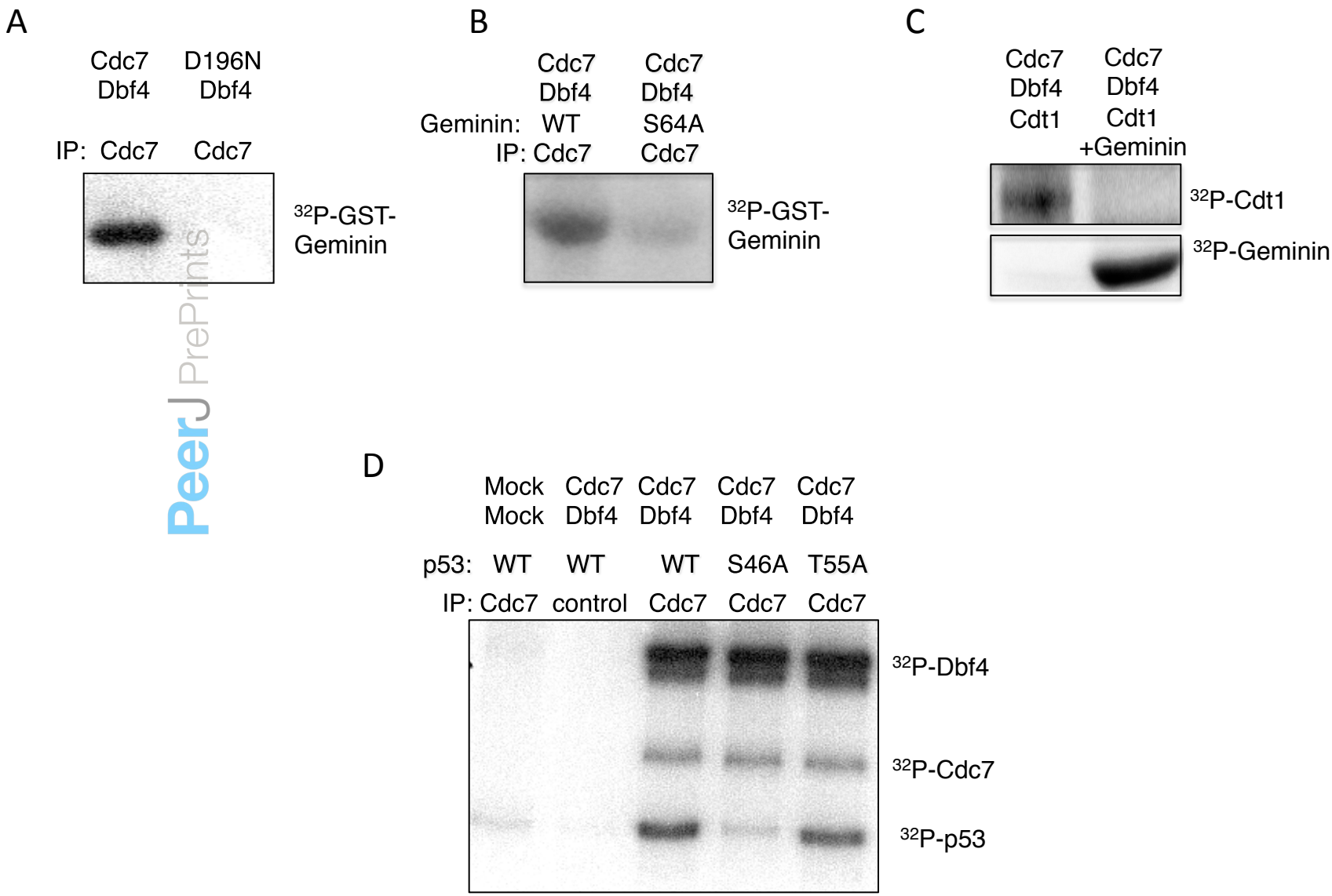

Figure S4