

## Report

# The SCF/Slimb Ubiquitin Ligase Limits Centrosome Amplification through Degradation of SAK/PLK4

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## Summary

Centrioles are essential for the formation of microtubule-derived structures, including cilia and centrosomes. Abnormalities in centrosome number and structure occur in many cancers and are associated with genomic instability [1]. In most dividing animal cells, centriole formation is coordinated with DNA replication and is highly regulated such that only one daughter centriole forms close to each mother centriole [1, 2]. Centriole formation is triggered and dependent on a conserved kinase, SAK/PLK4 [3–8]. Downregulation and overexpression of SAK/PLK4 is associated with cancer in humans, mice, and flies [9–11]. Here we show that centrosome amplification is normally inhibited by degradation of SAK/PLK4 degradation, mediated by the SCF/Slimb ubiquitin ligase. This complex physically interacts with SAK/PLK4, and in its absence, SAK/PLK4 accumulates, leading to the striking formation of multiple daughter centrioles surrounding each mother. This interaction is mediated via a conserved Slimb binding motif in SAK/PLK4, mutations of which leads to centrosome amplification. This regulation is likely to be conserved, because knockout of the ortholog of Slimb,  $\beta$ -Trcp1 in mice, also leads to centrosome amplification [12]. Because the SCF/ $\beta$ -Trcp complex plays an important role in cell-cycle progression, our results lead to

new understanding of the control of centrosome number and how it may go awry in human disease.

## Results and Discussion

Two ubiquitin ligases, the SKP1-CUL1-F-box-protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C), ubiquitylate many cell-cycle regulators, assuring irreversibility in cell-cycle transitions [13, 14]. Previous work suggested the SCF complex and protein degradation to be important players in the control of centrosome number [15–18]. These complexes comprise three polypeptides—Skp1 (SkpA in *Drosophila*), Cullin1/3, and Roc1/Rbx1—and a fourth variable adaptor that recognizes substrates, the F-box protein [13, 19]. Skp1 and Cul1 localize to the centrosome [17]. Furthermore, depletion of SkpA and the F-box protein, Slimb in *Drosophila* and its ortholog  $\beta$ -Trcp1 in mice, leads to a dramatic increase in centrosome number and abnormal mitotic spindles [12, 15, 16]. However, these results may not reflect direct regulation of centriole number but merely the diverse roles of the SCF complex in cell-cycle control [13]. This led us to investigate the role of protein degradation in centriole biogenesis, and in particular, the role of the SCF/Slimb complex.

We first investigated changes in centrosome number in cultured *Drosophila* cells after depletion of *Slimb* and *SkpA*. We used *Geminin*, an inhibitor of licensing of DNA replication, as a positive control for changes in centrosome number. This is because depletion of *Geminin* in human cells leads to DNA rereplication, associated with centrosome amplification [20]. Indeed, *Geminin* RNAi led to an increase in the proportion of cells with more than two centrosomes in *Drosophila* cells (Figures S1A and S1B available online) and large nuclei as expected after DNA rereplication (Figures S1A, S2A, and S2B). RNAi of *Slimb* and *SkpA* in the same time-frame resulted in changes in cell-cycle profile (Figures S1C and S1D) and a similar increase in the proportion of cells with more than two centrosomes (Figures S1A and S1B). An increase in centrosome number after *Slimb* and *SkpA* depletion could result from aborted mitosis, aborted cytokinesis, or a block in S phase, and so be independent of direct changes in the centrosome duplication machinery. The different cell-cycle profiles seen after downregulation of *SkpA* and *Slimb* (Figure S1D) are likely to reflect the diverse roles played by multiple SkpA-containing SCF complexes in the degradation of several cell-cycle regulators [13]. Those differences and the lack of any significant increase in the proportion of mitotic (not shown) and polyploid cells after *Slimb* depletion (Figures S1D and S2B) suggest that the observed increase in centrosome number is unlikely to arise indirectly because of abnormal cell-cycle progression. We therefore conclude that downregulation of *SkpA* or *Slimb* is likely to lead to impaired degradation of a common substrate that regulates centriole duplication with consequent centrosome amplification. Strikingly, depletion of *Slimb* led to a very specific phenotype at the electron microscope level (Figure 1A). We observed more than one daughter centriole budding from a single mother, which is not normally seen in cycling cells, where there is strict limit to one single daughter

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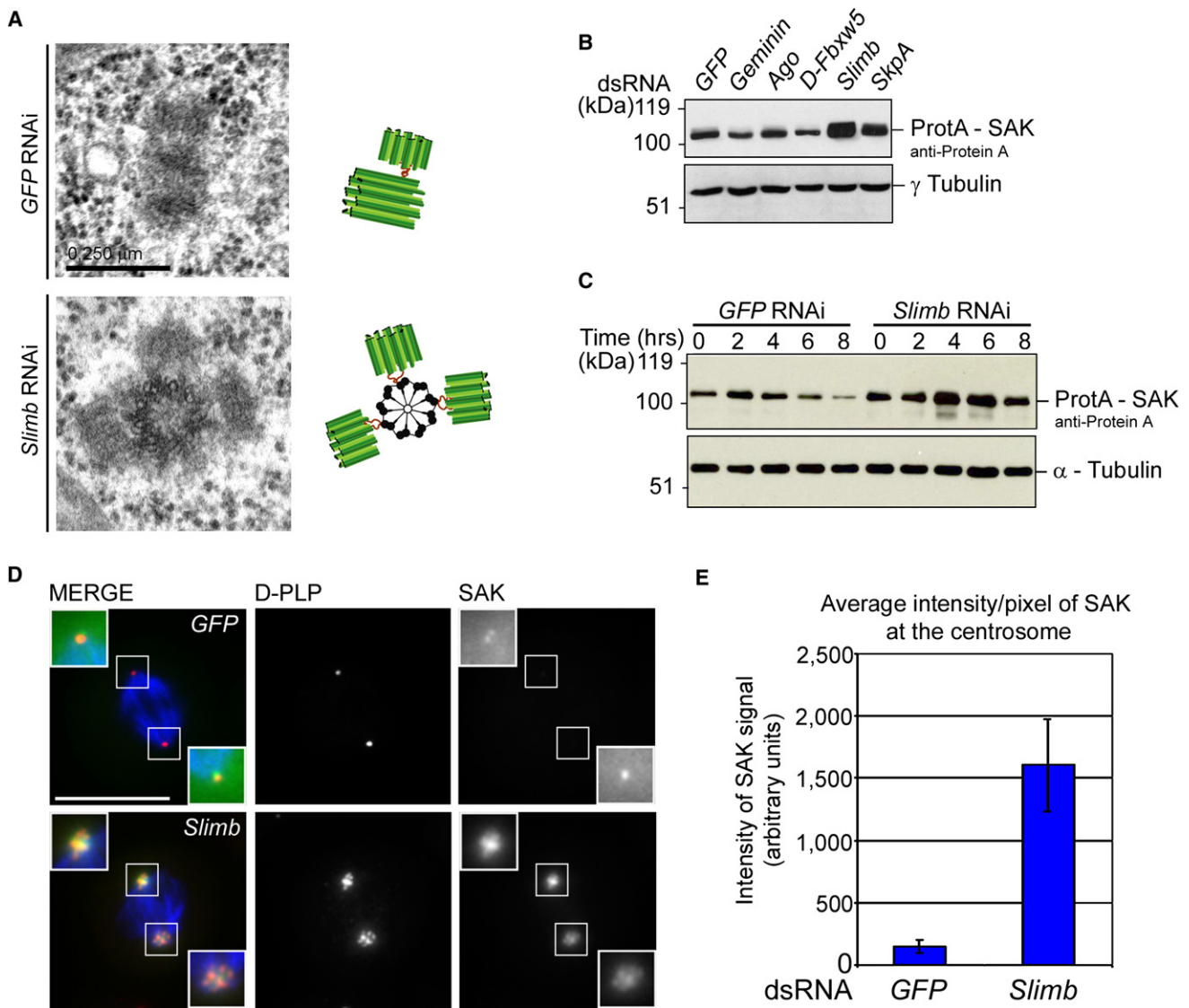


Figure 1. SAK/PLK4 Degradation Is Mediated by the SCF/Slimb-E3 Ubiquitin Ligase

(A) Transmission electron micrographs (TEM) reveal the growth of more than one procentriole close to the mother centriole in cells depleted from *Slimb*, but not in the *GFP* RNAi control. Scale bar represents 0.250  $\mu\text{m}$ .

(B) RNAi for two components of the SCF/Slimb ubiquitin ligase complex (*SkpA*, *Slimb*) stabilizes SAK/PLK4 protein. pMT-ProtA-SAK cells were transfected with dsRNAs; SAK/PLK4 expression was induced for 15 hr before harvesting. Cells were not synchronized. Note that RNAi of two other F-box proteins (*Ago* and *D-Fbxw5*) or *Geminin* did not lead to an increase in SAK/PLK4 levels.

(C) *Slimb* RNAi stabilizes SAK/PLK4 protein. pMT-ProtA-SAK cells were transfected with dsRNA and induced for 15 hr (see Supplemental Experimental Procedures). Cells were washed at time zero and harvested at the indicated time points.

(D and E) SAK/PLK4 endogenous levels at centrosomes were measured in metaphase cells after *Slimb* and *GFP* RNAi. SAK/PLK4 (green), D-PLP (Pericentriole Like Protein, red; centrosome marker),  $\alpha$ -tubulin (blue). All images were acquired with the same light intensity and camera exposure. Raw images are shown. Insets show the centrosome area (1.8 $\times$  magnification), where contrast has been optimized for purposes of showing the presence of SAK/PLK4 signal. Scale bar represents 10  $\mu\text{m}$ .

per mother centriole. A similar phenotype is also observed upon overexpression of SAK/PLK4 and SAS-6 [3, 7, 21], a molecule known to be downstream of SAK/PLK4 and to induce centrosome amplification upon overexpression [5, 21, 22]. Those rosette-like structures are reminiscent to the ones observed in multiciliated epithelial cells, which express high levels of SAK/PLK4 and SAS-6 [23, 24], and where as many as 300 centrioles/basal bodies are formed per cell.

We investigated whether the SCF/Slimb complex was targeting SAK/PLK4 or SAS-6 for degradation by asking whether their protein levels were higher in the absence of

*Slimb* or *SkpA*. Because the levels of endogenous SAK/PLK4 are extremely low and difficult to detect by western blotting [6, 7], we used a stable cell line in which SAK/PLK4 was fused with Protein A and could be expressed from the inducible metallothionein promoter. We investigated levels of SAK/PLK4 after induction of its expression by culture in medium containing  $\text{CuSO}_4$  for 15 hr. SAK/PLK4 levels were considerably higher after downregulation of *SkpA* and *Slimb*, but not of *Geminin* or other F-box proteins (Figure 1B; Figure S2C). The increase in levels of SAK/PLK4 that we see after downregulation of the SCF/Slimb complex contrasts with the lack of

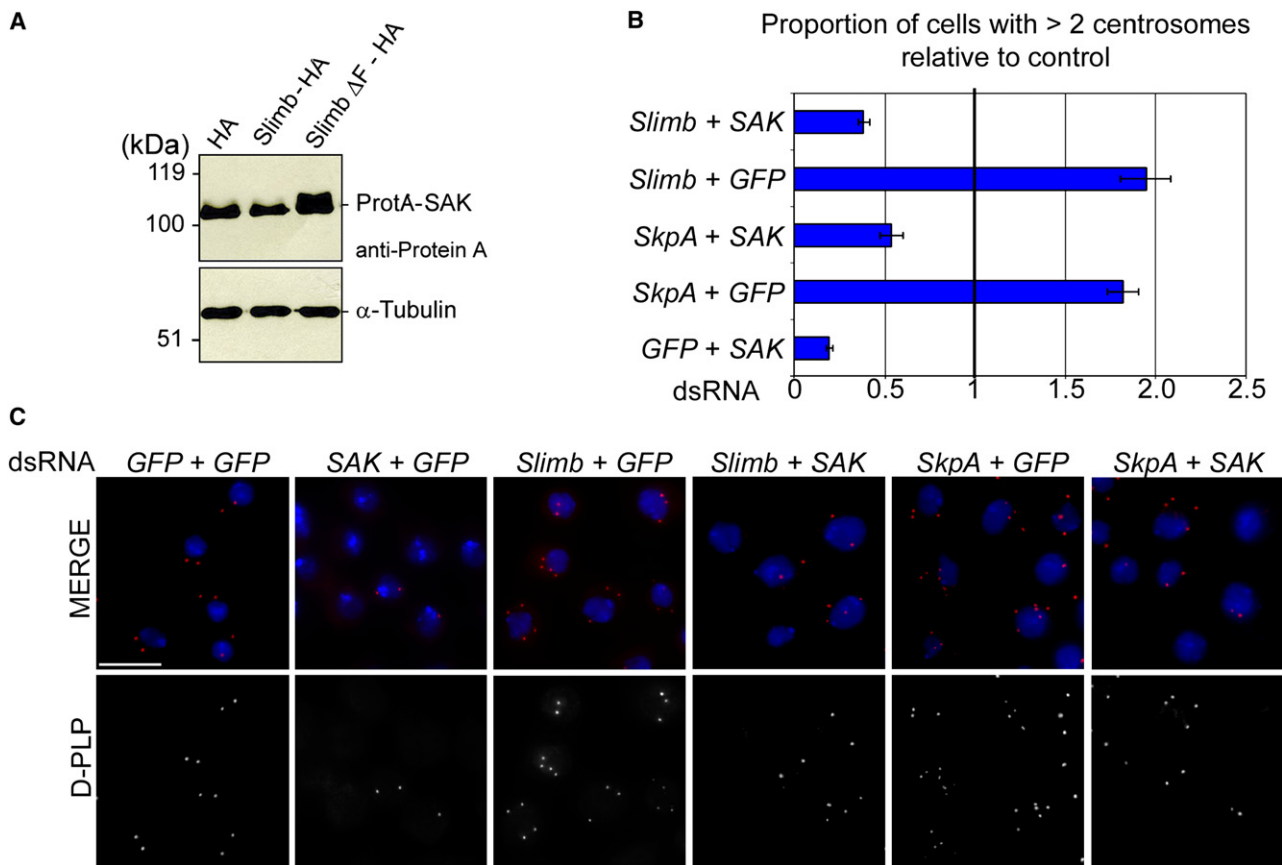


Figure 2. Centrosome Amplification upon *Slimb* RNAi is Dependent on SAK/PLK4

(A) Expression of a *Slimb* mutant lacking the F-box (*Slimb*  $\Delta$ F) leads to accumulation of SAK/PLK4 protein. pMT-ProtA-SAK cell line was transiently transfected with act5-HA, act5-*Slimb*-HA, or act5-*Slimb*  $\Delta$ F-HA constructs and cell extracts were prepared after a 15 hr induction of SAK/PLK4 expression. Controls for transfection were performed with an HA antibody (not shown).

(B and C) Centrosome amplification observed after depletion of *Slimb* and *SkpA* is dependent on SAK/PLK4.

(B) Cells were treated with dsRNA (indicated) and assayed for centrosome numbers. Quantitation of supranumerary centrosomes after RNAi. Results were normalized relative to controls (ratio is equal to 1 in *GFP* controls). Data are the average of three RNAi experiments  $\pm$  SEM ( $n = 200$  cells in each experiment).

(C) D-PLP (red) and DNA (blue). Scale bar represents 10  $\mu$ m.

effect on the levels of SAS-6 protein (Figure S3). This result is consistent with a recent report that human SAS-6 is degraded by the APC/Cdh1 [21] and suggests that the centrosome amplification observed after *Slimb* depletion is due to impaired SAK/PLK4 degradation.

We further investigated the regulation of SAK/PLK4 levels by the SCF/Slimb complex. We followed SAK/PLK4 levels during a period of 8 hr after induction of its expression for 15 hr, with subsequent removal of CuSO<sub>4</sub> prior to the time course. Whereas SAK/PLK4 degradation had commenced 4 hr after removal of CuSO<sub>4</sub> from control cells, its degradation began only after 8 hr in *Slimb*-depleted cells (Figure 1C). We verified this result investigating the endogenous SAK/PLK4 by developing a sensitive immunocytochemical detection system for this protein. SAK/PLK4 levels and localization might be cell-cycle regulated, as they are for other members of the polo kinase family, so we made all comparisons by examining cells at the same cell-cycle stage (metaphase). *Slimb* depletion resulted in an increase in the levels of the endogenous SAK/PLK4 at the centrosome and the whole cell, and thus our results are unlikely to reflect abnormal localization properties of SAK/PLK4 (Figures 1D and 1E; Figures S4 and S5A). This is also unlikely to be an indirect consequence of an increase

in the number of centrosomes at each pole after *Slimb* RNAi, because we also saw more intense SAK/PLK4 staining without any increase in the size of poles (Figure S5B, upper pole). This suggests that the centrosome amplification seen after *Slimb* RNAi results from an increase in SAK/PLK4 levels. To further verify these results, we used a dominant-negative approach. *Slimb* binds to *SkpA* through its F-box and mutants in this domain have been shown to impair SCF/Slimb function [25]. Indeed, we observed elevated levels of the SAK/PLK4 protein after expression of such a mutant (Figure 2A), which is accompanied by more than a 2-fold increase in the proportion of cells with more than two centrosomes ( $68.2 \pm 1.1$  after transfection of *Slimb*  $\Delta$ F-HA versus  $32.3 \pm 2.5$  with HA alone).

If *Slimb* is targeting SAK/PLK4 for degradation, then the increase in centrosome number seen after impairment of *Slimb* function should depend on SAK/PLK4. We performed double RNAi of SAK/PLK4 and either *Slimb* or *SkpA*. We observed a reduction in centrosome number after both *Slimb* and SAK/PLK4 RNAi, and after both *SkpA* and SAK/PLK4 RNAi, as compared to *Slimb* and *SkpA* alone, suggesting that the phenotype observed after depletion of the *Slimb* and *SkpA* is dependent on the presence of SAK/PLK4, corroborating our hypothesis (Figures 2B and 2C).

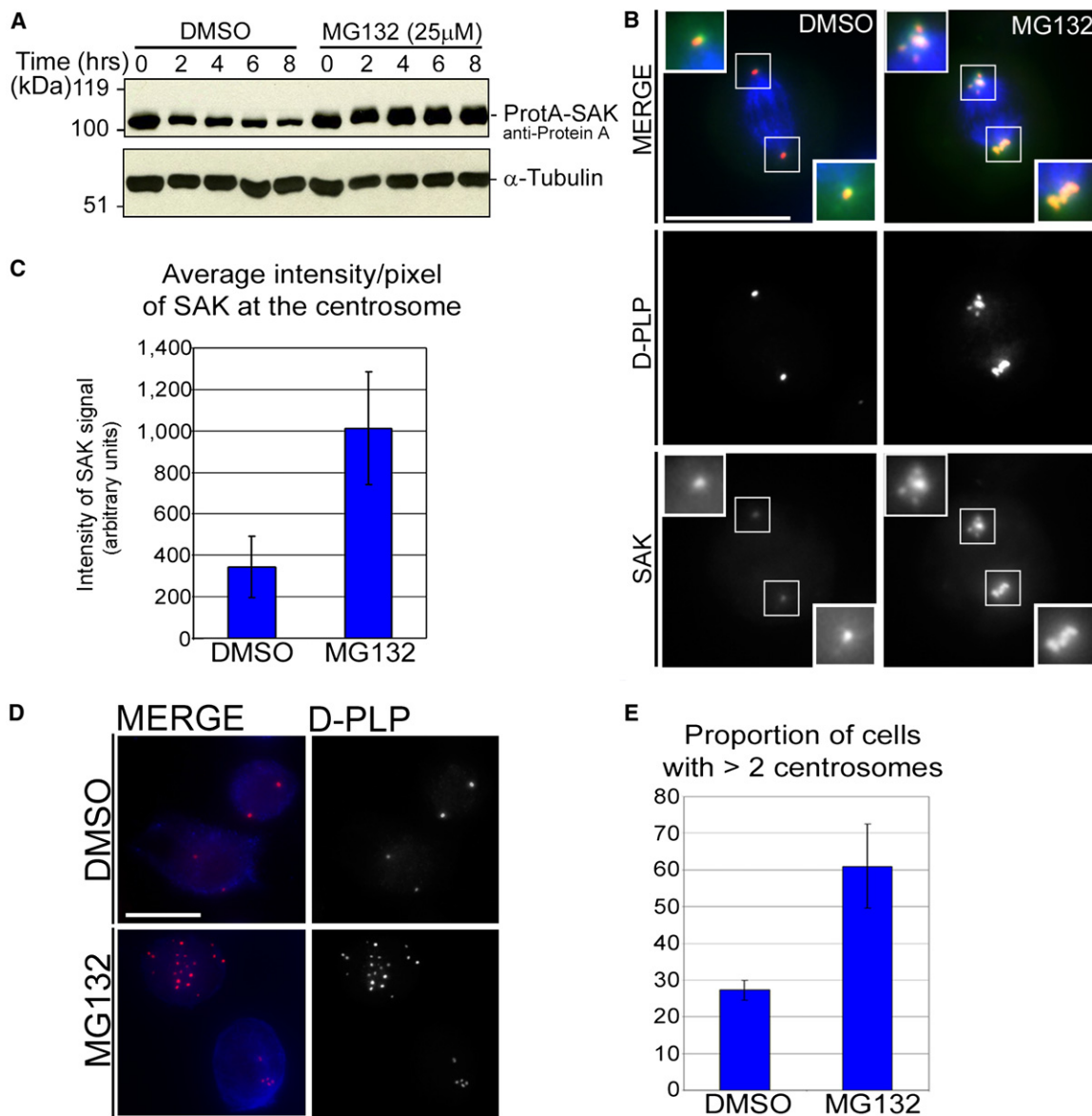


Figure 3. SAK/PLK4 Is Degraded by the 26S Proteasome

(A) Proteasome inhibition stabilizes SAK/PLK4 protein. pMT-ProtA-SAK cells were induced for 15 hr with CuSO<sub>4</sub>, after which they were washed and incubated with vehicle (DMSO) or the proteasome inhibitor MG132. Cells were harvested at the indicated time points.

(B and C) Wild-type cells were treated with MG132 for 6 hr and stained for endogenous SAK (green),  $\alpha$ -tubulin (blue), and D-PLP (red).

(B) Images were acquired and are shown as in Figure 1D. Scale bar represents 10  $\mu$ m.

(C) Quantitation of the average intensity of SAK/PLK4 signal per pixel at the centrosomes as in Figure 1E.

(D and E) Treatment with MG132 led to centrosome amplification.

(D) Cells were fixed and stained for  $\alpha$ -tubulin (blue) and D-PLP (red).

(E) The proportion of cells in interphase with more than two centrosomes was counted. Note that control *Drosophila* tissue-culture cells show a background population with more than two centrosomes (approximately 30%) as reported before [34].

SCF ubiquitin ligases tag proteins with a polyubiquitin chain that targets them for degradation by the 26S proteasome [19]. We tested whether SAK/PLK4 is degraded by the 26S proteasome. We observed degradation of SAK/PLK4 in the absence but not in the presence of MG132, a 26S proteasome inhibitor, for 8 hr (Figure 3A). We observed an approximate 3-fold increase in the levels of endogenous SAK/PLK4 at the metaphase centrosome when we inhibited proteasome activity (Figures 3B and 3C). This was also reflected in the total centrosome number, as cells accumulated centrosomes after such MG132 treatment (Figures 3D and 3E). Together, these

results strongly suggest that SAK/PLK4 is degraded by the proteasome and that impairment of this regulation leads to centrosome amplification.

F-box proteins are known to bind directly to SCF substrates through a WD40 repeat motif [13]. To determine whether there is an association between SAK/PLK4 and Slimb, we built two cell lines in which these proteins were tagged with Protein A. We used immunoglobulin-coated beads to purify each of these proteins and the proteins associated with them. Complexes were subjected to SDS-PAGE and individual bands identified by mass spectrometry. We were able to identify

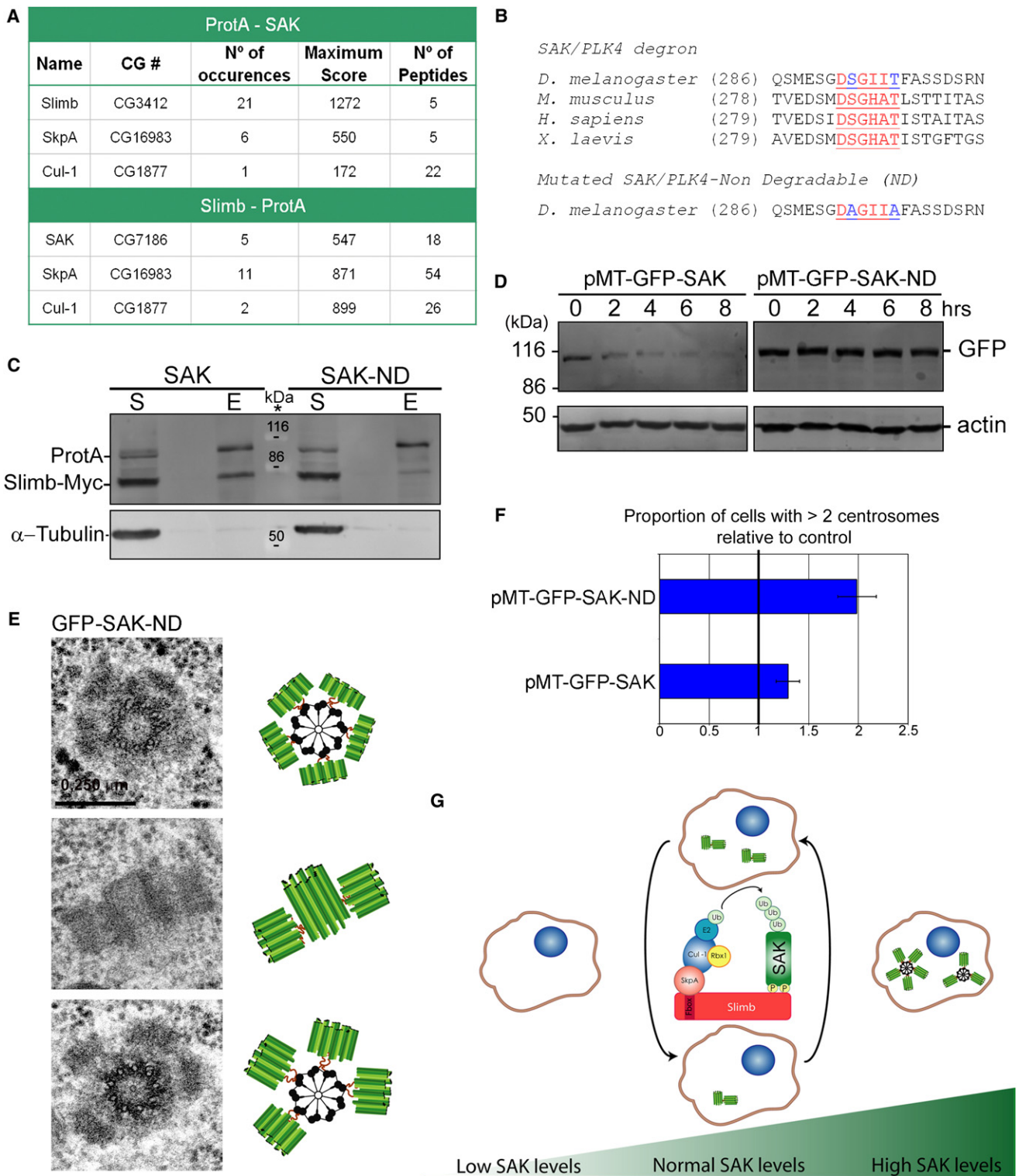


Figure 4. The SCF/Slimb Ubiquitin Ligase Interacts with SAK/PLK4 to Mediate Its Degradation

(A) SAK/PLK4 interacts with Slimb. Pull-down of ProtA-SAK and Slimb-ProtA fusion proteins, followed by SDS-PAGE and sequencing of approximately 20–30 excised bands. The number of times each interacting protein appeared, the maximum MASCOT score, and the number of identified peptides for each interacting protein are indicated. These interactions have been reproduced at least five times in independent experiments.

(B) Consensus degron in SAK/PLK4 sequence for Slimb recognition. Position of first amino acid residue shown in sequence is indicated in brackets. Degron is indicated in red and mutated sites are in blue.

(C) Western blot of pull-down of ProtA from cell lines expressing both ProtA-SAK (inducible) and Slimb-Myc (constitutive) or both ProtA-SAK-ND (inducible) and Slimb-Myc (constitutive). S, input/supernatant after cell lysis; E, bead eluate; protein ladder is in middle lane (marked by asterisk). Western was first

members of the Slimb/SCF complex, including the subunits Slimb, SkpA, and Cul-1, after pull-down of SAK/PLK4 (Figure 4A). Conversely, endogenous SAK/PLK4 was identified in the pull-down of protein-A-tagged Slimb (Figure 4A). The pull-down included other proteins (data not shown) among which was Period, a known interactor of Slimb [25]. We have used a cell line expressing protein A as a negative control and did not find any of those molecules (data not shown). Moreover, we have performed similar experiments to purify other cell-cycle protein complexes and have never identified SAK/PLK4 (data not shown).

Substrates of the SCF/Slimb complex show a conserved degron that is recognized by Slimb [13]. We found that SAK/PLK4 protein has a modified Slimb recognition site in *Drosophila* (DSGIIT; position 293) and humans (DSGHAT; position 285) (Figure 4B). In previous studies, human SAK/PLK4 was shown to be ubiquitinated [26] and removal of a region encompassing amino acids 272–311 led to stabilization of the human SAK/PLK4, although no analysis was performed on centrosome number [27]. We mutated the conserved Slimb recognition site to DAGIIA in *Drosophila* SAK/PLK4, which we called SAK-ND (nondegradable). This mutation abolished SAK's ability to interact with Slimb (Figures 4B and 4C; Figure S6A) and led to a decrease in SAK's ubiquitinated species (Figure S6B). Moreover, SAK-ND is more stable in comparison to its WT counterpart (Figure 4D). Thus, this mutation allowed us to test the biological significance of SAK/PLK4 degradation by the SCF/Slimb complex. Upon expression of the mutated SAK/PLK4 construct (Figure 4E), we observed a very similar phenotype to the one registered after *Slimb* RNAi (Figure 1A), i.e., rosette-like structures. These structures contained centriole precursors (Figure 4E, top) or elongating centrioles (Figure 4E, middle and bottom). Those results suggest a common centriole-amplification phenotype observed after *Slimb* RNAi and after expression of the SAK-ND mutant. The observation of at least five centrioles clustered at spindle poles in mitosis suggests that some of the supranumerary centrioles formed in one cycle after impairment of SAK/PLK4 degradation by the SCF/Slimb complex become proper microtubule-organizing centers (Figure S6C). We quantified the formation of supranumerary centrosomes at the light microscope level. Upon transient low-level expression of the SAK-ND construct fused to GFP, we systematically observed a statistically significant increase in centrosome number in transfected cells compared to cells expressing low levels of the WT SAK fused to GFP (Figure 4F; Figure S6D). Indeed, expression of low levels of SAK-ND led to a 2-fold increase in centrosome amplification (Figure 4F). This is comparable to the effect of *Slimb* RNAi (Figure S1B) and after expression of a dominant-negative form of Slimb (referred to above),

suggesting that high levels of SAK/PLK4 underlie those phenotypes.

Together, our results show that degradation of SAK/PLK4 by the SCF/Slimb complex is critical to restrict its function, preventing centrosome amplification. Although other mechanisms for regulation of SAK/PLK4 activity may exist, proteolytic degradation is likely to be conserved across species and be relevant in the animal. The Slimb-binding phosphodegron in SAK/PLK4 is conserved in vertebrates (Figure 4B). Moreover, knockout of the ortholog of Slimb in mice,  $\beta$ -*Trcp1*, and both *SkpA* and *Slimb* *Drosophila* mutants show an increase in centrosome number [12, 15, 16], as do flies that overexpress SAK/PLK4 [4, 5, 10].

It has been suggested that centriole overduplication is normally prevented by a "licensing event," the disengagement of centrioles that occurs at the exit of mitosis, required for the next duplication cycle [28, 29]. Requirement for this event ensures that centrioles duplicate only once in every cell cycle. However, it was recently shown that centriole amplification can also occur through the simultaneous formation of many daughters from a single mother, as it is after overexpression of SAK/PLK4 [3–5, 7] and SAS-6 [21] or inhibition of proteolysis by the proteasome [18]. Our data suggests that proteolysis mediated by SCF/Slimb plays an important role in limiting the amount of SAK/PLK4, but not SAS-6, available to trigger multiple daughter formation. When SAK/PLK4 is not degraded, multiple daughter centrioles may be generated, which led to centrosome amplification (Figures 4E, 4F, and 4G). The regulation of centriole number thus emerges as a multistep mechanism, where proteolysis controls the activity of key players, SAK/PLK4 and SAS-6 [18, 21].

The mammalian F-box counterpart of Slimb,  $\beta$ -Trcp, plays a role in the DNA damage response by halting the cycle in response to genotoxic inputs [30–32]. Misregulation of  $\beta$ -Trcp has been observed in cancer cells [13, 33] in which centrosome number is often increased after stress [1]. It is thus possible that Slimb/ $\beta$ -Trcp coordinates checkpoints that monitor the status of DNA replication with centrosome number. The misregulation of this F-box protein would therefore result in changes in levels of SAK/PLK4, which are associated with mitotic abnormalities and oncogenesis [6, 9, 10]. Given that other substrates of the Slimb/ $\beta$ -Trcp F-box protein require to be phosphorylated and our own results with the SAK-ND mutant (Figures 4C–4E), it is likely that SAK/PLK4 also requires such a modification at the serine and threonine residues in its DSGIIT degron to mark it for degradation. Future research on the identity and regulation of the kinase that phosphorylates SAK/PLK4 and on the localization of Slimb and SAK/PLK4 phosphorylated at the DSGIIT degron should indicate how different signaling events are

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probed for Myc to detect Slimb-Myc and then for protein A to show SAK and SAK-ND.  $\alpha$ -tubulin was used as a loading control. Note that both cell lines express similar levels of Slimb-Myc and ProtA-SAK or ProtA-SAK-ND (see Figure S6A), but that the latter brings down much less Slimb-Myc.

(D) Cells were transfected transiently with pMT-GFP-SAK and pMT-GFP-SAK-ND and induced for 15 hr (see Supplemental Experimental Procedures). Cells were washed at time zero, split into different wells, and harvested at the indicated time points. Note the stabilization of SAK-ND.

(E) Transmission electron micrographs (TEM) of GFP-SAK-ND cells reveal the growth of multiple procentrioles around a single mother. Scale bar represents 0.250  $\mu$ m.

(F) Cells transfected transiently with pMT-GFP-SAK and pMT-GFP-SAK-ND, with no CuSO<sub>4</sub> induction, were stained for centrosomes (D-PLP) for counts (see Figure S6D for images). The proportion of cells with more than two centrosomes in interphase cells was counted and normalized over controls (ratio is equal to 1 in GFP-only transfected controls). Data are the average of three experiments  $\pm$  SEM. The increase in the population of cells with more than two centrosomes after SAK-ND transfection is statistically different from the one observed after transfection of WT SAK/PLK4 (odds ratio of 3.5;  $p < 10^{-15}$ , Pearson's  $\chi^2$  test)

(G) Regulation of the levels of SAK/PLK4 by the SCF/Slimb complex. The levels of SAK/PLK4 have to be very well controlled. Overexpression of SAK/PLK4, or depletion of the SCF/Slimb complex, which led to SAK/PLK4 accumulation, results in centrosome amplification through simultaneous multiple procentriole formation.

coordinated in the cell to prevent centrosome amplification. Our results open new avenues for understanding the mechanisms underlying centrosome misregulation that are of direct relevance to human disease.

#### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at [http://www.current-biology.com/supplemental/S0960-9822\(08\)01554-6](http://www.current-biology.com/supplemental/S0960-9822(08)01554-6).

#### Acknowledgments

We thank Z. Carvalho-Santos, V. Archambault, M. Przewlaka, N. Dzhindzhev, and P. D'Avino for help with experiments. We thank D. Calderon, I. Ederly, T.D. Murphy, L. Capalbo, M. Savoian, J. Rocha, G. Rogers, and S. Rogers for reagents. We thank the IGC imaging unit, in particular N. Moreno for help with image acquisition and quantitation. We thank M. Godinho, R. Martinho, V. Archambault, J. Pereira-Leal, Z. Carvalho-Santos, and N. Sepúlveda for helpful discussions on the manuscript. We would like to thank G. Rogers and S. Rogers for sharing unpublished data. We are grateful to grants from Cancer Research UK, Fundação Calouste Gulbenkian, Fundação para a Ciência e Tecnologia (FCT; POCI2010, PTDC/SAU\_OBD/73194/2006), Câmara Municipal de Oeiras, an EMBO Installation Grant to M.B.-D., and to an International Joint Project Grant from the Royal Society for collaboration between the M.B.-D. and D.M.G. groups. I.C.-F., A.R.-M., and I.B. are recipients of scholarships from FCT. The authors declare no competing financial interests.

Received: August 14, 2008

Revised: November 11, 2008

Accepted: November 13, 2008

Published online: December 11, 2008

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