

# Plk4 *trans*-autophosphorylation regulates centriole number by controlling $\beta$ TrCP-mediated degradation

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## Summary

Centrioles are the main constituents of the mammalian centrosome and act as basal bodies for ciliogenesis. Centrosomes organize the cytoplasmic microtubule network during interphase and the mitotic spindle during mitosis, and aberrations in centrosome number have been implicated in chromosomal instability and tumor formation. The centriolar protein Polo-like kinase 4 (Plk4) is a key regulator of centriole biogenesis and is crucial for maintaining constant centriole number, but the mechanisms regulating its activity and expression are only beginning to emerge. Here, we show that human Plk4 is subject to  $\beta$ TrCP-dependent proteasomal degradation, indicating that this pathway is conserved from *Drosophila* to human. Unexpectedly, we found that stable overexpression of kinase-dead Plk4 leads to centriole overduplication. This phenotype depends on the presence of endogenous wild-type Plk4. Our data indicate that centriole overduplication results from disruption of Plk4 *trans*-autophosphorylation by kinase-dead Plk4, which then shields endogenous Plk4 from recognition by  $\beta$ TrCP. We conclude that active Plk4 promotes its own degradation by catalyzing  $\beta$ TrCP binding through *trans*-autophosphorylation (phosphorylation by the other kinase in the dimer) within homodimers.

**Key words:** Plk4, Autophosphorylation,  $\beta$ TrCP, Centriole duplication

## Introduction

Centrioles are the main constituents of the mammalian centrosome and act as basal bodies for ciliogenesis (Nigg and Raff, 2009). Centrosomes organize the cytoplasmic microtubule network during interphase and the mitotic spindle during mitosis, and aberrations in centrosome number have been implicated in chromosomal instability (Ganem et al., 2009) and tumor formation (Nigg and Raff, 2009; Zyss and Gergely, 2009). Furthermore, mutations in genes coding for centriolar and/or centrosomal proteins are linked to a variety of human diseases, notably brain diseases and ciliopathies (Nigg and Raff, 2009). Thus, centrosome assembly as well as centriole biogenesis and duplication are crucial processes requiring accurate control (Bornens, 2002; Doxsey et al., 2005; Strnad and Gonczy, 2008; Nigg and Raff, 2009). A central role in the control of centriole biogenesis and duplication has been attributed to Polo-like kinase 4 (Plk4; also known as SAK) (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Nigg, 2007). Echoing earlier studies in invertebrate model organisms (Kirkham et al., 2003; Delattre et al., 2006; Pelletier et al., 2006; Kilburn et al., 2007; Nakazawa et al., 2007; Rodrigues-Martins et al., 2007; Dammermann et al., 2008; Song et al., 2008), Plk4-induced centriole biogenesis in human cells involves the sequential assembly of several essential proteins, including human Sas-6, Cep135, CPAP (human Sas-4) and CP110 (Kleylein-Sohn et al., 2007). Recent studies have shown that the levels of *Drosophila* Plk4 are regulated by the ubiquitin-proteasome pathway through the E3 ubiquitin ligase SCF<sup>Slimb</sup> $\beta$ TrCP (SKP1–CUL1–F-box protein; Slimb and  $\beta$ TrCP are homologous F-box proteins in *Drosophila* and humans, respectively) (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Here, we have addressed the issue of how Plk4 stability is controlled in human cells. In particular, we have explored a possible relationship between Plk4 phosphorylation and  $\beta$ TrCP-dependent

degradation. Our results lead us to conclude that Plk4 undergoes autophosphorylation *in trans* and that this modification is crucial for Plk4 stability and the maintenance of a constant centriole number.

## Results and Discussion

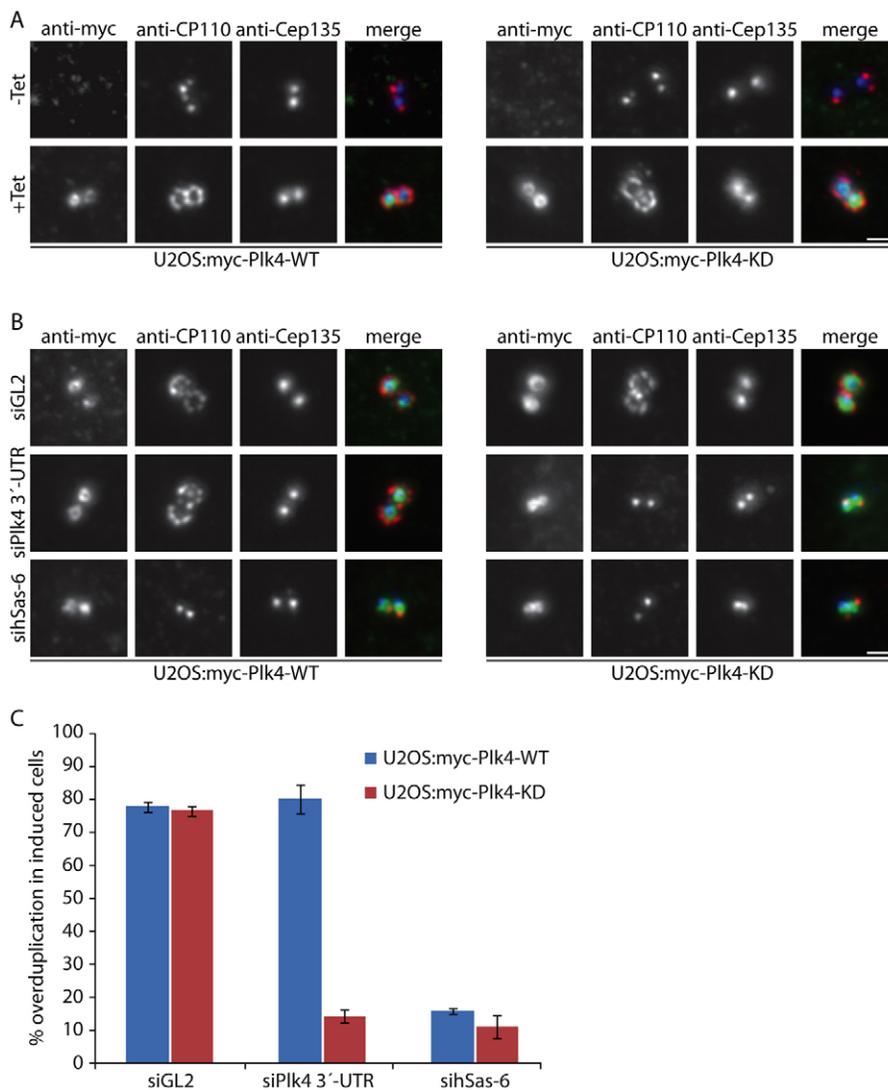
To complement a previously described U2OS cell line that allows the tetracycline-inducible expression of myc-tagged wild-type Plk4 (U2OS:myc–Plk4-WT) (Kleylein-Sohn et al., 2007), we generated a comparable cell line for the expression of myc-tagged kinase-dead Plk4, which is incapable of autophosphorylation (U2OS:myc–Plk4-KD; supplementary material Figs S1, S2). When we compared the ability of wild-type Plk4 and kinase-dead Plk4 to induce centriole overduplication, we observed robust centriole overduplication in both cell lines (Fig. 1A). This observation was unexpected, because centriole overduplication induced by transient transfection of Plk4 had previously been reported to depend on kinase activity (Habedanck et al., 2005; Sillibourne et al., 2010). These early results have been confirmed both in this study (supplementary material Fig. S3) and elsewhere (Holland et al., 2010). Therefore, we conclude that stable overexpression of Plk4-KD produces more extensive centriole overduplication than transient transfection. Possibly, higher expression levels of Plk4-KD produce pleiotropic effects, such as displacement of endogenous Plk4 from centrioles.

To understand how Plk4-KD induces centriole overduplication, we first carried out siRNA rescue experiments to determine whether this phenotype depends on endogenous Plk4. U2OS:myc–Plk4-WT and U2OS:myc–Plk4-KD cells were transfected for 24 hours with siRNA oligonucleotides targeting the 3'-untranslated region (siPlk4 3'-UTR) or control oligonucleotides (siGL2) and then arrested in aphidicolin before myc–Plk4 (WT or KD) expression

was induced. As expected, the transfection of control siRNA duplexes did not inhibit Plk4-induced centriole overduplication in either cell line (Fig. 1B,C). Likewise, 80% of cells overexpressing myc-Plk4-WT still exhibited centriole overduplication even after depletion of endogenous Plk4 (Fig. 1B, left panel; Fig. 1C). By stark contrast, centriole overduplication was reduced to 14% of cells upon concomitant expression of myc-Plk4-KD with siPlk4 3'-UTR (Fig. 1B, right panel; Fig. 1C). A similar reduction of centriole overduplication was observed when either myc-Plk4-WT or myc-Plk4-KD were overexpressed in cells lacking human Sas-6 (Fig. 1B,C), as expected (Kleylein-Sohn et al., 2007). These results demonstrate that myc-Plk4-KD is only able to induce centriole overduplication in the presence of endogenous wild-type Plk4.

While this work was in progress, *Drosophila* Plk4 was shown to be degraded in an SCF<sup>Slimb/βTrCP</sup>-dependent manner (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Consequently, depletion of the SCF ubiquitin ligase Slimb (mammalian βTrCP) led to stabilization of Plk4 and to centriole overduplication (Cunha-Ferreira et al., 2009; Rogers et al., 2009). As expected for a proteasome-dependent degradation mechanism, human Plk4 protein levels also increased upon proteasome inhibition with 1 μM MG132

for 16 hours (supplementary material Fig. S4) and similar results were independently reported by others (Holland et al., 2010; Sillibourne et al., 2010). This prompted us to speculate that Plk4-KD might cause centriole overduplication by interfering with the βTrCP-mediated degradation of endogenous Plk4. To explore this notion, we first investigated whether human Plk4 protein levels are also controlled by βTrCP. Asynchronously growing U2OS cells were depleted of βTrCP by siRNA transfection and centriole numbers monitored by immunofluorescence microscopy. Upon depletion of βTrCP, Plk4 protein levels at the centrosome increased about sevenfold compared with those of control cells (Fig. 2A,B). Moreover, βTrCP-depleted cells exhibited centriole overduplication, partially in a rosette-like arrangement of procentrioles, reminiscent of Plk4 overexpression in human cells (Kleylein-Sohn et al., 2007) and earlier work in *Drosophila* (Cunha-Ferreira et al., 2009; Rogers et al., 2009). To directly demonstrate a role of Plk4 in the observed phenotype, we analyzed the effects of βTrCP depletion in the absence of Plk4. Whereas 48% of βTrCP-depleted control cells exhibited overduplicated centrioles, virtually no centriole overduplication was observed after co-depletion of βTrCP and Plk4, similar to results observed after depletion of Plk4 alone (Fig. 2A,C). Instead, these latter treatments increased the proportion of

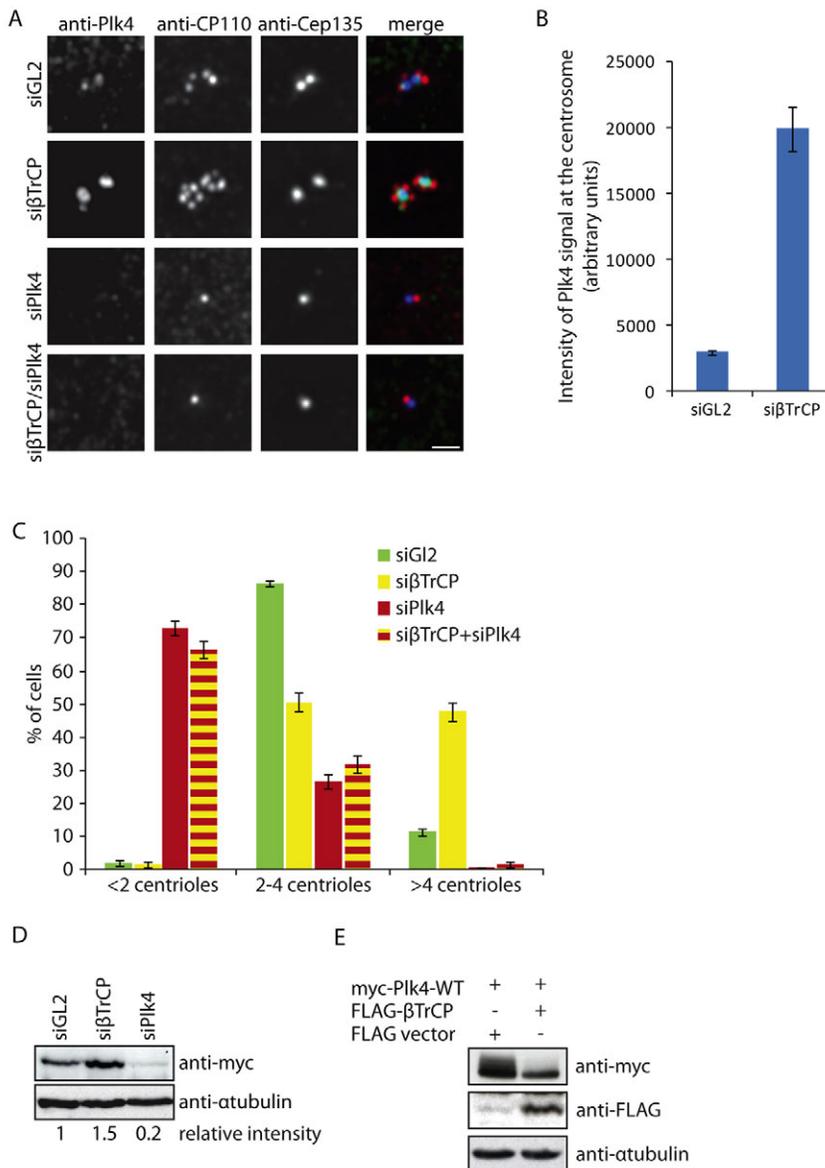


**Fig. 1. Kinase-dead Plk4 causes centriole overduplication.** (A) U2OS:myc-Plk4-WT or U2OS:myc-Plk4-KD cells were arrested with aphidicolin for 24 hours before expression of myc-Plk4-WT or myc-Plk4-KD was induced for 16 hours. No tetracycline was added to controls. Cells were fixed and stained with antibodies for the myc epitope (green), CP110 (red) and Cep135 (blue). (B) U2OS:myc-Plk4-WT or U2OS:myc-Plk4-KD cells were transfected for 24 hours with siRNA oligonucleotides targeting *GL2*, the 3'-UTR of *Plk4* or human *Sas-6* prior to induction of Plk4 expression (myc-Plk4-WT or myc-Plk4-KD) for 16 hours. Cells were stained for the myc epitope (green), CP110 (red) and Cep135 (blue). (C) Percentage of cells, treated as described in B, that exhibited centriole overduplication. Data from three independent experiments ( $n=100$ ) are shown. Error bars denote s.e.m. Scale bars: 1 μm.

cells with fewer than two centrioles to 67% and 73%, respectively (Fig. 2C). Hence, the centriole-overduplication phenotype produced by depletion of  $\beta$ TrCP clearly requires Plk4. To demonstrate that  $\beta$ TrCP modulates levels of Plk4 protein, we depleted  $\beta$ TrCP for 72 hours before inducing expression of myc-Plk4-WT for the last 24 hours of siRNA treatment. Compared with cells treated with control siRNA duplexes (siGL2), depletion of  $\beta$ TrCP led to a 1.5-fold increase in Plk4-WT protein (Fig. 2D). Also, *Plk4* siRNA treatment (carried out for control) abolished Plk4 expression, as expected (Fig. 2D). Conversely, coexpression of  $\beta$ TrCP and Plk4-WT in 293T cells led to a decrease in Plk4 protein (Fig. 2E). Together, the above data demonstrate that  $\beta$ TrCP modulates Plk4 protein levels in human cells and thus contributes to the maintenance of correct centrosome number. This confirms and extends earlier work in *Drosophila* (Cunha-Ferreira et al., 2009; Rogers et al., 2009) and shows that the  $\beta$ TrCP-Plk4 pathway is conserved in *Drosophila* and mammals (see also Guardavaccaro et al., 2003; Holland et al., 2010; Sillibourne et al., 2010). Yet another recent study also demonstrates centriole overduplication in U2OS cells upon

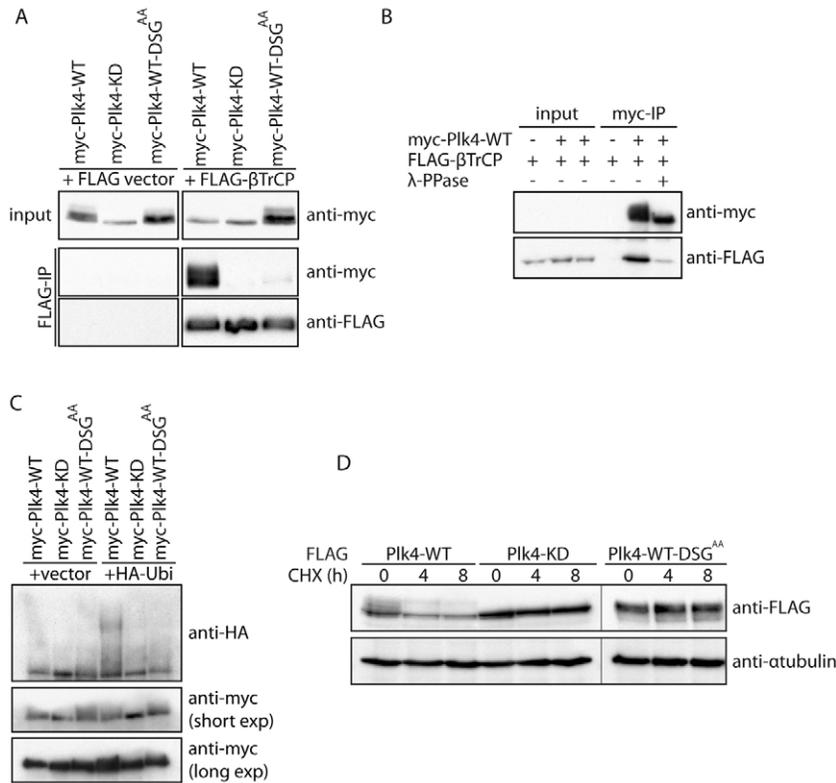
depletion of the SCF component Cul1, although a role for  $\beta$ TrCP was not emphasized (Korzeniewski et al., 2009).

To further explore our proposition that Plk4-KD might cause centriole overduplication by interfering with the degradation of endogenous (active) Plk4, we next investigated whether Plk4-KD is able to bind to  $\beta$ TrCP. Usually,  $\beta$ TrCP binds its substrates via a DSGxx[S/T] motif (DSG motif) in the substrate protein and this interaction is thought to be regulated by phosphorylation of two phospho-acceptor sites (S/T) within this so-called phosphodegron (Nakayama and Nakayama, 2006). Human Plk4 carries an evolutionarily conserved DSG motif spanning residues 284 to 289 (DSGHAT). Indeed, an interaction between human Plk4-WT and  $\beta$ TrCP could readily be demonstrated by co-immunoprecipitation and, as predicted, this interaction required an intact DSG motif (Fig. 3A; supplementary material Fig. S5). Both [serine/threonine]-to-alanine (Plk4-WT-DSG<sup>AA</sup>) and [serine/threonine]-to-glutamate (Plk4-WT-DSG<sup>DD</sup>) substitutions at positions 285 and 289 disrupted the interaction of Plk4 with  $\beta$ TrCP (Fig. 3A; supplementary material Fig. S5), indicating that aspartate did not mimic phosphorylation



**Fig. 2. Plk4 protein levels are controlled by  $\beta$ TrCP.**

(A) U2OS cells were transfected for 72 hours with siRNA oligonucleotides targeting *GL2*, *βTrCP*, *Plk4* or *βTrCP* and *Plk4* before cells were stained for Plk4 (green), CP110 (red) and Cep135 (blue). Scale bar: 1  $\mu$ m. (B) Plk4 signal intensity was measured in cells treated as described in A. Data of three independent experiments ( $n=30$ ) are shown. Error bars denote s.e.m. (C) Percentage of cells, treated as described in A, with the indicated number of centrioles; the number of centrioles was counted via CP110 staining. Data of three independent experiments ( $n=100$ ) are shown. Error bars denote s.e.m. (D) U2OS:myc-Plk4-WT cells were transfected for 72 hours with siRNA oligonucleotides targeting *GL2*, *βTrCP* or *Plk4*. Myc-Plk4-WT expression was induced during the last 24 hours of siRNA treatment. Then, cells were harvested and analyzed for myc-Plk4-WT expression by immunoblotting against the indicated proteins. The myc-signal was normalized against the  $\alpha$ -tubulin signal and quantified with ImageJ. (E) Myc-Plk4-WT was expressed in 293T cells together with FLAG vector or FLAG- $\beta$ TrCP. Cells were harvested and protein levels analyzed by immunoblotting.



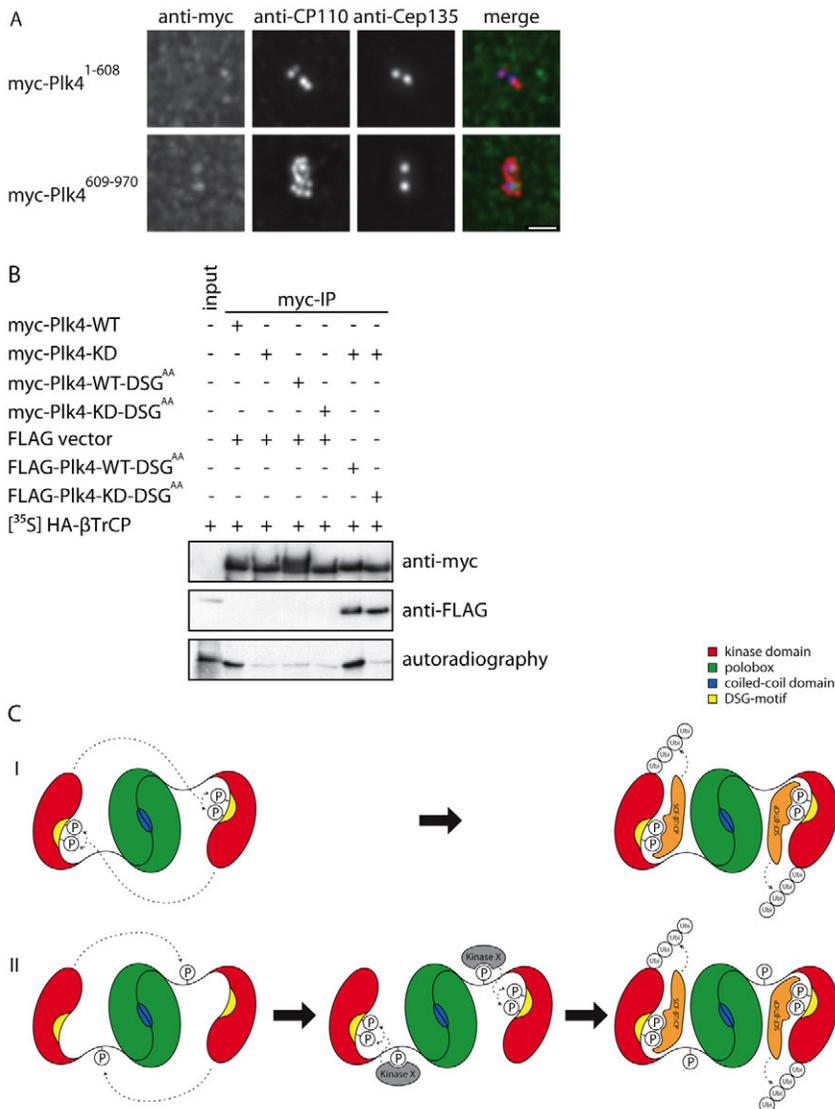
**Fig. 3. Kinase-dead Plk4 is resistant to  $\beta$ TrCP-mediated degradation.** (A) FLAG- $\beta$ TrCP was coexpressed with myc-Plk4-WT, myc-Plk4-KD or myc-Plk4-WT-DSG<sup>AA</sup> in 293T cells. Cell extracts were subjected to anti-FLAG immunoprecipitations and immunoprecipitates were probed for the indicated proteins by immunoblotting. (B) FLAG- $\beta$ TrCP and myc-Plk4-WT were coexpressed in 293T cells. Anti-myc immunoprecipitations were performed and immunoprecipitates treated with  $\lambda$ -phosphatase ( $\lambda$ PPase) where indicated. The co-immunoprecipitated proteins were detected by immunoblotting. (C) Myc-Plk4-WT, myc-Plk4-KD or myc-Plk4-DSG<sup>AA</sup> were coexpressed for 24 hours with HA vector or HA-ubiquitin. Cell extracts were subjected to anti-myc immunoprecipitations and probed by immunoblotting for the indicated proteins. (D) FLAG-Plk4-WT, FLAG-Plk4-KD and FLAG-WT-DSG<sup>AA</sup> were expressed in 293T cells before protein synthesis was blocked by cycloheximide. Cells were harvested at the indicated time points (hours) and protein levels analyzed by immunoblotting.

in this context. Importantly, under the exact same experimental conditions, Plk4-KD did not interact with  $\beta$ TrCP, strongly suggesting that Plk4 activity is required for this interaction. Confirming this conclusion, the Plk4- $\beta$ TrCP complex could be disrupted by  $\lambda$ -phosphatase ( $\lambda$ PPase) treatment (Fig. 3B). Furthermore, Plk4-KD was ubiquitylated less efficiently than Plk4-WT and in this regard resembled Plk4-WT-DSG<sup>AA</sup> (Fig. 3C). Consistent results were obtained *in vivo* (Fig. 3C) and *in vitro* (supplementary material Fig. S6), arguing against co-precipitation of other ubiquitylated proteins with Plk4-WT. One would expect that lack of ubiquitylation should stabilize Plk4 by protecting it from degradation via the 26S proteasome. Indeed, whereas Plk4-WT was degraded in cells treated with cycloheximide, Plk4-KD was stabilized to a similar extent as was Plk4-WT-DSG<sup>AA</sup> (Fig. 3D). Together, these data suggest that Plk4 kinase activity is necessary for its interaction with  $\beta$ TrCP and, consequently, its polyubiquitylation and subsequent degradation. Overall, these results are in good agreement with two recent studies that independently demonstrate a role of Plk4 autophosphorylation in controlling Plk4 stability (Holland et al., 2010; Sillibourne et al., 2010). Interestingly, Holland and co-workers observed only partial stabilization of a DSG-phosphodegrogen mutant, whereas more extensive stabilization was observed for a version of Plk4 that lacked a stretch of 24 residues comprising this motif (Holland et al., 2010). This observation prompted the authors to propose that a second,  $\beta$ TrCP-independent, pathway might also contribute to the regulation of Plk4 stability (Holland et al., 2010).

The finding that Plk4-KD cannot interact with  $\beta$ TrCP argues against the possibility that excess Plk4-KD causes centriole overduplication through sequestration of  $\beta$ TrCP. This led us to explore an alternative model involving dimerization of Plk4. As shown previously, Plk4 dimerizes via its C-terminal coiled-coil region (Leung et al., 2002; Habedanck et al., 2005). This

dimerization is independent of kinase activity, as confirmed here by co-immunoprecipitation experiments (supplementary material Fig. S7). Several Plk4 fragments differing in their ability to dimerize were overexpressed in U2OS cells and assayed for their ability to trigger centriole overduplication. As shown in supplementary material Figs S8-S10, Plk4<sup>1-608</sup> is clearly active as a kinase and interacts with  $\beta$ TrCP but does not dimerize owing to truncation of its C terminus, whereas Plk4<sup>609-970</sup> is able to dimerize with Plk4-WT but does not interact with  $\beta$ TrCP owing to truncation of the kinase domain. Remarkably, Plk4<sup>609-970</sup> caused strong centriole overduplication, occasionally resulting in a rosette-like arrangement of procentrioles, whereas Plk4<sup>1-608</sup> failed to do so (Fig. 4A). This reinforces the view that excess Plk4-KD is able to cause centriole overduplication, provided that its ability to dimerize with endogenous Plk4 is preserved.

The above data led us to conclude that excess Plk4-KD triggers centriole overduplication by virtue of its ability to (hetero-)dimerize with endogenous, active Plk4. If this is the case, the Plk4-KD polypeptide could potentially be phosphorylated *in trans* by the Plk4-WT polypeptide (but not vice versa), and phosphorylated Plk4-KD could then sequester SCF <sup>$\beta$ TrCP</sup> by acting as a decoy. A corollary of this model is that autophosphorylation *in trans* should convert Plk4-KD to a  $\beta$ TrCP-binding species. To test this prediction, we expressed various combinations of myc- or FLAG-tagged Plk4 proteins differing in their activity status (WT or KD) and/or ability to be recognized by  $\beta$ TrCP (DSG-WT or DSG<sup>AA</sup>). In these experiments, the myc-tagged constructs served as bait for  $\beta$ TrCP binding, whereas the FLAG-tagged constructs, competent to dimerize with Plk4 but incompetent to bind  $\beta$ TrCP, provided kinase activity. The ability of the immunoprecipitated complexes to bind to  $\beta$ TrCP was then analyzed via an *in vitro* binding assay. Coexpression of FLAG-Plk4-KD-DSG<sup>AA</sup> with myc-Plk4-KD failed to restore  $\beta$ TrCP binding, as expected, considering the



**Fig. 4. Plk4 autophosphorylation in *trans* catalyzes βTrCP binding.** (A) U2OS cells were transfected with myc-Plk4<sup>1-608</sup> or myc-Plk4<sup>609-970</sup> for 48 hours. Cells were stained for the myc epitope (green), CP110 (red) and Cep135 (blue). Scale bar: 1 μm. (B) 293T cells were transfected with the indicated plasmids. Anti-myc immunoprecipitates were incubated with in-vitro-translated [<sup>35</sup>S]-methionine-labeled HA-βTrCP in an in vitro binding assay. The co-immunoprecipitated proteins were analyzed by immunoblotting and autoradiography. (C) Two schematic models. According to model I, Plk4 autophosphorylation directly phosphorylates the DSG motif in *trans* and this is sufficient for βTrCP binding. Alternatively (model II), Plk4 autophosphorylation in *trans* creates a docking site for an unknown kinase X, which then phosphorylates the DSG motif. In both cases, phosphorylation of the DSG motif is proposed to initiate the degradation of Plk4. Ubi, ubiquitin.

absence of *trans*-autophosphorylation. By stark contrast, coexpression of FLAG-Plk4-WT-DSG<sup>AA</sup> with myc-Plk4-KD fully restored the binding of myc-Plk4-KD to βTrCP (Fig. 4B). This demonstrates that autophosphorylation in *trans* is required to confer βTrCP-binding properties to Plk4. In excellent agreement with this conclusion, Plk4-WT was independently shown to promote destruction of Plk4-KD through intermolecular phosphorylation (Holland et al., 2010).

Whether autophosphorylation is not just required, but is sufficient for Plk4-βTrCP binding is not presently known. A priori, it is possible that Plk4 *trans*-autophosphorylation directly activates the phosphodegron for βTrCP binding (Fig. 4C, model I). Alternatively, Plk4 might autophosphorylate in *trans* on sites distinct from the phosphodegron that then serve to recruit a different kinase X, which in turn phosphorylates Plk4 on the phosphodegron or in close proximity to this motif (Fig. 4C, model II). In support of this latter possibility, we emphasize that degradation of several βTrCP targets, e.g. β-catenin (Liu et al., 2002), Wee1 (Watanabe et al., 2004) and Erp1 (Liu and Maller, 2005; Rauh et al., 2005; Hansen et al., 2006), involves recruitment of phosphodegron-directed kinases through phosphorylation-dependent docking sites.

In conclusion, our study shows that autophosphorylation controls βTrCP-mediated degradation of Plk4. In line with observations on the activation-dependent degradation of other protein kinases (Kang et al., 2000; Lu and Hunter, 2009), we propose that active Plk4 catalyzes its own degradation and that this provides a tight coupling between activity status and protein abundance. We further show that Plk4 degradation involves autophosphorylation in *trans*, and this provides a rationale for the observation that excess Plk4-KD can trigger centriole overduplication through a mechanism requiring endogenous, active Plk4. Our data provide not only important mechanistic insight into the regulation of Plk4, but also raise interesting new questions. Most importantly, future research should aim at exploring the timing of Plk4 degradation during the cell cycle and the identification of a putative kinase X that is proposed here to contribute to control Plk4 stability.

## Materials and Methods

### Plasmids and antibodies

Cloning of *Plk4* and *βTrCP1* cDNA has been described previously (Habedanck et al., 2005; Chan et al., 2008). Sequence mutations in Plk4 were inserted by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions using the following primers: Plk4 S285A/T289A 5'-GAAGACTCAA-

TTGATGCTGGGCATGCCGCAATTCTACTGC-3'; Plk4 S285D/T289D 5'-GAA-GACTCAATTGATGACGGGCATGCCGCAATTCTACTGC-3'. HA-ubiquitin was generously provided by Stefan Müller (Max-Planck Institute of Biochemistry, Martinsried, Germany).

An anti-Plk4 monoclonal antibody (IgG1) was generated against recombinant MBP-Plk4 (AA715-970) purified from *Escherichia coli*. Anti-myc (9E10) (Evan et al., 1985), anti-CP110 (Schmidt et al., 2009), anti-CAP350 (Yan et al., 2006), anti-C-Nap1 (Fry et al., 1998) and anti-Cep135 (Kleylein-Sohn et al., 2007) antibodies have been described previously. Anti- $\alpha$ -tubulin (Sigma-Aldrich), anti-FLAG (Sigma-Aldrich) and anti-HA (Covance) antibodies were commercially obtained. To simultaneously visualize different polyclonal rabbit antibodies, these were directly labeled by Alexa-Red-555 and Alexa-Cy5-647 fluorophores, using the corresponding Antibody Labeling Kits (Invitrogen).

#### Cell culture and transfections

Transient transfections of 293T cells were performed using TransIT-LT1 transfection reagent (Mirus Bio) according to the manufacturer's protocol.

The tetracycline-inducible U2OS myc-Plk4-WT cell line (U2OS:myc-Plk4-WT) has been described previously (Kleylein-Sohn et al., 2007). A tetracycline-inducible cell line expressing myc-tagged kinase dead Plk4 (U2OS:myc-Plk4-KD) was generated by transfection of U2OS T-REx cells (Invitrogen). Stable transformants were established by selection for 2 weeks with 1 mg ml<sup>-1</sup> G418 (Invitrogen) and 50  $\mu$ g ml<sup>-1</sup> hygromycin (Merck). U2OS cells were cultured as described previously (Habedanck et al., 2005) and myc-Plk4 expression was induced by the addition of tetracycline (1  $\mu$ g ml<sup>-1</sup>).

#### siRNA-mediated protein depletion

Plk4 was depleted using the previously described siRNA-duplex oligonucleotides targeting the coding sequence (Habedanck et al., 2005) or the 3'-UTR of *Plk4* (5'-CTCCTTTCAGACATATAAG-3'). Human Sas-6 was depleted using the siRNA-duplex oligonucleotides previously described (Kleylein-Sohn et al., 2007).  $\beta$ TrCP1 and  $\beta$ TrCP2 were depleted using siRNA-duplex oligonucleotides targeting both paralogs (Guardavaccaro et al., 2003). Luciferase duplex GL2 was used for control (Elbashir et al., 2001). Transfections were performed using Oligofectamin (Invitrogen) according to the manufacturer's protocol.

#### Cell-extract preparation and biochemical assays

At 24 hours post-transfection, 293T cells were collected and washed in PBS and lysed on ice for 30 minutes in lysis buffer [50 mM Tris-HCl, pH 7.4, 0.5% IgePal, 150 mM NaCl, 1 mM DTT, 5% glycerol, 50 mM NaF, 1 mM PMSF, 25 mM  $\beta$ -glycerophosphate, 1 mM vanadate, Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics)]. Lysates were cleared by centrifugation for 15 minutes at 13,000 g at 4°C.

To assay protein-degradation kinetics, translation was inhibited by the addition of 25  $\mu$ g/ml cycloheximide for the indicated time.

For immunoprecipitations, the extracts were incubated with protein-G beads (GE Healthcare) and 10  $\mu$ g of the appropriate antibodies for 1.5 hours at 4°C. Immunocomplexes bound to beads were washed three times with wash buffer (lysis buffer with 300 mM NaCl). Bound proteins were eluted by boiling in 2 $\times$  SDS sample buffer, resolved by SDS-PAGE and analyzed by immunoblotting.

For in vitro binding assays, the washed immunocomplexes were suspended in lysis buffer and incubated for 1.5 hours at 4°C with HA- $\beta$ TrCP, which had been in vitro translated using the TNT-T7 quick coupled transcription/translation system (Promega) with [<sup>35</sup>S]-methionine according to the manufacturer's protocol. After washing three times with wash buffer, the bound proteins were eluted by boiling in 2 $\times$  SDS sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting and autoradiography.

In vitro ubiquitylation of in-vitro-translated [<sup>35</sup>S]-methionine-labeled Plk4 was carried out using a HeLa-lysate-based ubiquitin-conjugation kit (Enzo Life Sciences) according to the manufacturer's protocol. Conjugation was visualized by immunoblotting and autoradiography.

In vitro kinase assays using immunoprecipitated Plk4 were carried out at 30°C in kinase buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 5% glycerol, 1 mM DTT). Reactions were stopped after 30 minutes by addition of sample buffer. Samples were then analyzed by immunoblotting and autoradiography.

#### Microscopic techniques

Cells were fixed in methanol for 5 minutes at -20°C. Antibody incubations and washings were performed as described previously (Meraldi et al., 1999). Stainings were analyzed using a DeltaVision microscope on a Nikon TE200 base (Applied Precision), equipped with an APOPLAN 100 $\times$ /1.4 N.A. oil-immersion objective. Serial optical sections obtained 0.2- $\mu$ m apart along the z-axis were processed using a deconvolution algorithm and projected into one picture using Softworx. For quantitation of Plk4 levels at the centrosome with ImageJ, z-stacks from control and treated samples were acquired with the same exposure and maximum-intensity projections were carried out. Background signal intensity was subtracted from Plk4 signal intensity.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/13/2163/DC1>

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