Control of Centriole Numbers by Plk4 Autophosphorylation and βTrCP-mediated Degradation

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1 SUMMARY

Proper centrosome numbers are imperative for faithful cell division, as aberrant centrosome numbers can lead to chromosomal instability, a hallmark of cancer development (Nigg 2002; Ganem *et al.,* 2009). Hence, initiation of centriole duplication has to be tightly regulated. Recently, we and others demonstrated that Polo-like kinase 4 (Plk4) fulfills a pivotal role in regulating this process (Bettencourt-Dias *et al.,* 2005; Habedanck *et al.,* 2005). Plk4 protein levels and its activity directly correlate with centriole numbers: depletion of Plk4 leads to sequential loss of centrioles in successive cell divisions (Bettencourt-Dias *et al.,* 2005; Habedanck *et al.,* 2005) and its overexpression promotes *bona fide* overduplication of centrioles (Habedanck *et al.,* 2005; Kleylein-Sohn *et al.,* 2007), while both lead to progressive increase in abnormal spindle formation (reviewed in Nigg 2007). Even though Plk4 is a key regulator of centriole biogenesis and is crucial for maintaining constant centriole number, the mechanisms regulating its activity and expression are only beginning to emerge.

Here, we show that human Plk4 is subject to βTrCP-dependent proteasomal degradation, indicating that this pathway is conserved from *Drosophila* to human (Cunha-Ferreira *et al.,* 2009; Rogers *et al.,* 2009). Unexpectedly, we found that stable overexpression of kinase-dead Plk4 leads to centriole overduplication. Our data indicate that this phenotype depends on the presence of endogenous wild-type Plk4 and that centriole overduplication results from disruption of Plk4 *trans*-autophosphorylation by kinase-dead Plk4, which then shields endogenous Plk4 from recognition by βTrCP. We conclude that active Plk4 promotes its own degradation by catalyzing βTrCP binding through *trans*-autophosphorylation within homodimers which has been independently confirmed by others (Holland *et al.,* 2010). Additionally, we propose that Plk4 autophosphorylation is not sufficient for its degradation and that instead an additional kinase is required for this process.

2 INTRODUCTION

The centrosome, Latin for "central body", was first discovered in the late 19th century by Édouard van Beneden in various parasites (van Beneden 1875-6; van Beneden 1883). While van Beneden discovered centrosomes and described them at a morphological level, it was Theodor Boveri who coined the term centrosome and postulated that the centrosome is self-replicating (Boveri 1887; Boveri 1888). Moreover, he later formulated the hypothesis that centrosome and chromosome aberrations are linked and contribute to tumorigenesis (Boveri 1914). Even though centrosomes are present in almost all eukaryotes, their composition, organization, mode of replication and specific functions have remained elusive until the rediscovery of centrosome biology in the late 20th century. Today, pivotal functions of the centrosome have been uncovered and described, albeit the details of how these functions are fulfilled and regulated are still under intense investigation. Centrosome function is twofold, as microtubule-organizing center (MTOC) in dividing cells and as scaffold for basal bodies of flagella or cilia in differentiated or quiescent cells. In recent years, centrosome biology has become widely recognized due to the causal link between centrosome aberrations and the development of various human diseases.

2.1 Structure and Function of the Centrosome

2.1.1 Structure of the Centrosome

The centrosome is a non-membranous organelle of approximately 1 µm in diameter which is usually located in close proximity to the nucleus (reviewed in Doxsey 2001). It is composed of two interconnected centrioles which are highly stable, barrel-shaped arrays of microtubule triplets arranged in a nine-fold symmetry (Figure 1). The individual microtubules (MTs) of each triplet are referred to as the A-, B- and C-tubule and reach a length of 400 nm during centriole elongation (reviewed in Bornens 2002; Bettencourt-Dias and Glover 2007). In contrast to the A- and B-tubules, which span the complete proximal-distal axis of a fully grown centriole, the C-tubule does not stretch to the distal end of the centriole.

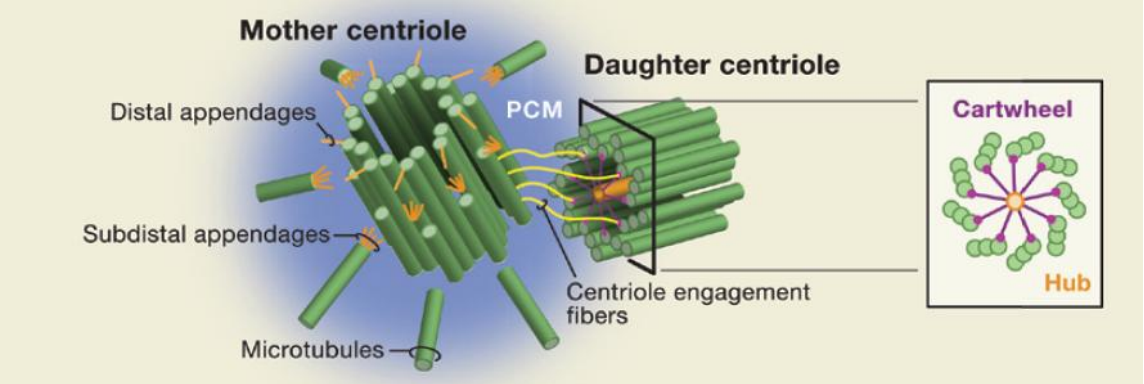

Figure 1. Centrosome and centriole structure. Schematic view of a centrosome containing mother and daughter centrioles. Both centrioles are composed of nine-fold microtubule (MT) triplets. In each triplet, the internal tubule is termed the A-tubule, followed by the B-tubule and C-tubule. The latter does not extend to the distal end of the centriole. The two centrioles are surrounded by the pericentriolar material (PCM), depicted in blue, and interconnected by an unknown linker (centriole engagement fibers) until disengagement at the exit from mitosis. The mature centriole carries subdistal and distal appendages, which dock cytoplasmic MTs and anchor the centriole at the plasma membrane to serve as basal body. The cartwheel structure depicted on the right has been suggested to serve as a template for procentriole formation (adapted from Bettencourt-Dias and Glover 2009).

The centrioles are embedded in the electron-dense, amorphous pericentriolar material (PCM), which harbors coiled-coil proteins that mediate protein-protein interactions (Doxsey 2001; Andersen *et al.*, 2003; Azimzadeh and Bornens 2007). Additionally, within the PCM proteins reside which are required for microtubule nucleation and anchoring as well as various cell cycle regulators (Moritz *et al.*, 1995; Zheng *et al.*, 1995; Moritz and Agard 2001). Centrioles and the PCM are intimately connected as loss of centrioles leads to dispersal of the PCM (Bobinnec *et al.*, 1998) and the PCM is *vice versa* required for the formation and stabilization of procentrioles (Dammermann *et al.*, 2004; Loncarek *et al.*, 2008).

Both centrioles present in a mammalian G1 phase cell are loosely tethered at their proximal ends by the proteins C-Nap1, rootletin and Cep68 (Fry *et al.*, 1998; Bahe *et al.*, 2005; Graser *et al.*, 2007b). Even though the two centrioles of a single centrosome are similar in their overall architecture, they are structurally and functionally distinct in that only one has fully matured (Piel *et al.*, 2000; Azimzadeh and Bornens 2007). Mature centrioles are characterized by the presence of two sets of appendages (distal and subdistal; Paintrand *et al.*, 1992) at their distal ends where they are attached to each of the nine centriolar MT doublets. Appendages have been shown to be involved in anchoring MTs and the centriole at the plasma membrane during ciliogenesis (Piel *et al.*, 2000; Azimzadeh and Bornens 2007) through characterization of several appendage proteins,

e.g. as ε-tubulin, Cep164, Cep170, ninein, and the ODF-2 splice variant hCenexin1 (Mogensen *et al.*, 2000; Chang *et al.*, 2003; Guarguaglini *et al.*, 2005; Ishikawa *et al.*, 2005; Graser *et al.*, 2007a; Soung *et al.*, 2009).

2.1.2 The Centrosome as the Microtubule-organizing Center (MTOC)

The most evident function of the centrosome lies in the orchestration of the microtubule network in eukaryotic cells as the microtubule organizing center (MTOC). Herein, the centrosome mediates the nucleation and anchoring of microtubules by the centrosome-associated γ-tubulin containing multiprotein ring complexes (γTuRCs). At the hub of the microtubule network, the centrosome is involved in the orchestration of cell motility, cell shape, cell adhesion, cell polarity and intracellular transport (reviewed in Doxsey 2001; Bornens 2002; Nigg 2004; Doxsey *et al.*, 2005; Azimzadeh and Bornens 2007; Bornens 2008). During cell division, the centrosome shapes the bipolar mitotic spindle to ensure faithful chromosome segregation (reviewed in Marshall 2009). The centrosome has also been attributed an essential function in asymmetric cell divisions, e.g. in stem cell divisions (Wang *et al.*, 2009). In contrast to the requirement for centrosomes as the MTOC in most eukaryotic cells, eukaryotes naturally lacking centrosomes have devised alternative mechanisms for spindle formation, as has been observed in higher plants and certain fungi (reviewed in Marshall 2009).

2.1.3 The Centriole as Template for Cilia and Flagella

Almost all eukaryotic cells form cilia at some point during their life cycle. Ciliogenesis begins when cells exit the cell cycle into a quiescent (G_0 phase) and/or differentiated state and the centrosome is translocated from the periphery of the nucleus to the plasma membrane (Figure 2). There, the centriole from which the cilium emanates is termed basal body. The mature basal body is anchored to the plasma membrane and serves as template for the outgrowth of the ciliary axoneme. *Vice versa*, cilia are resorbed and basal bodies are converted back to centrosomes when cells exit G_0 to re-enter the cell cycle. Importantly, while centrioles are not strictly required for mitosis, they are indispensable for ciliogenesis (reviewed in Pedersen and Rosenbaum 2008).

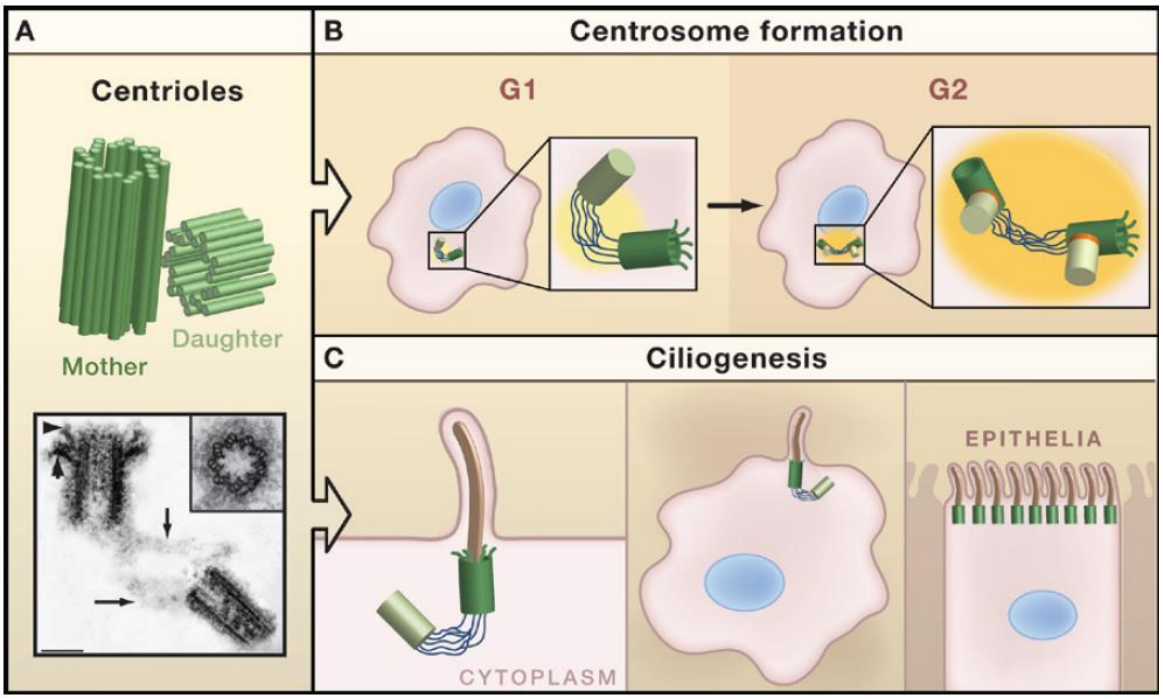

Figure 2. Centrioles form cilia and centrosomes. Schematic illustration of centrosome formation and ciliogenesis. **(A)** A G1 phase centrosome which consists of two centrioles that are loosely tethered by a fibrous network indicated by arrows in the EM micrograph. Note that the mature centriole carries distal and subdistal appendages (marked by arrowheads). The inset shows a cross-section of a centriole. **(B)** In proliferating cells, the parental centrioles (dark green) duplicate to give rise to two new centrioles (light green). **(C)** In quiescent cells the centrosome migrates to the cell surface where it is anchored at the plasma membrane and a cilium (brown) is assembled on the older parental centriole. Certain epithelial cells form a multiciliated surface from many centrioles (adapted from Nigg and Raff 2009).

Cilia are involved in a variety of cellular functions, ranging from cell motility, the reception of mechanical and chemical cues, brain development, signal transduction to transport duties in specialized tissues (reviewed in Gerdes *et al.*, 2009; Han and Alvarez-Buylla 2010). These very different functions can be fulfilled by a single organelle because cilia appear both as immotile, singular primary cilia and as motile cilia and flagella (reviewed in Dawe *et al.*, 2007). Ciliary morphology provides information about its function, as motile cilia are usually comprised of nine MT doublets, the A- and B-tubules of the basal body, which surround a central pair of single MTs (9+2), whereas immotile cilia lack the central MT pair and motor proteins (9+0; Satir and Christensen 2008). The beating of motile cilia is conferred by axonemal dynein which interconnects the outer MTs in cooperation with nexin (reviewed in Ibanez-Tallon *et al.*, 2003). Motile cilia enable the movement of whole organisms, in the case of *Paramecium*, or single cells within a multicellular organism, in the case of oocytes by multiciliated cells in the oviduct. Similarly, flagella enable the propulsion of the green algae *Clamydomonas* or spermatocytes. Immotile, single primary cilia on the other hand serve as transducers of

extracellular stimuli into intracellular signals (Satir and Christensen 2007; Gerdes *et al.*, 2009). This is accomplished by the accumulation of trans-membrane receptors in the ciliary membrane and the localization of downstream components of, for example, the Wnt and Shh signal transduction pathways to the cilium (reviewed in Michaud and Yoder 2006; Singla and Reiter 2006; Christensen and Ott 2007; Christensen *et al.*, 2007; Berbari *et al.*, 2009; Veland *et al.*, 2009).

Mutations in basal body- or cilium-associated genes result in malformed cilia or lack thereof and lead to a variety of pleiotropic diseases termed ciliopathies. These manifest themselves in a variety of disorders, for example Bardet-Biedl (Ansley *et al.*, 2003), Meckel-Gruber (Frank *et al.*, 2007), Joubert (Valente *et al.*, 2006) and Senior-Løken (Omran *et al.*, 2002) syndrome.

2.2 The Centrosome Cycle

Similar to chromosomes, the centrosome is duplicated during the cell cycle and the duplicated centrosomes are then divided among the daughter cells together with the segregated chromosomes. Cells do not have a checkpoint to stop the cell cycle in the presence of multiple centrosomes (Sluder *et al.*, 1997) and abnormal centrosome numbers severely interfere with bipolarity during mitosis. Therefore, cells duplicate their centrioles through a tightly regulated sequence of events termed the centrosome cycle, which is divided into four distinct phases: centriole duplication, maturation and elongation, centrosome separation and centriole disengagement (Figure 3).

At the onset of S phase the procentriole begins to form orthogonally to the proximal base of the parental centriole (Robbins *et al.*, 1968; Kuriyama and Borisy 1981; Vorobjev and Chentsov Yu 1982; Alvey 1985; Kochanski and Borisy 1990; Paintrand *et al.*, 1992). After elongation of the procentrioles during the following G2 phase, centrosome separation takes place by the severing of a physical linker connecting the two parental centrioles in response to phosphorylation of C-Nap1 and rootletin by Nek2 (Bahe *et al.*, 2005). Concomitantly, additional γ-tubulin ring complexes are recruited, leading to an increase in centrosome size and microtubule nucleation (Palazzo *et al.*, 2000). The separated centrosomes then travel to opposite poles of the cell, where they organize the bipolar mitotic spindle. During late M or early G1 phase the parental and daughter

centrioles disengage to lose their intimate connection and orthogonal orientation (Freed *et al.*, 1999; Piel *et al.*, 2000). Separase is thought to be involved in triggering the disengagement of the two centrioles (Tsou and Stearns 2006b), although the exact role of Separase in this process remains to be determined. The centrosome cycle is completed by a maturation step during G2 phase of the following cell cycle, in which the centriole formed during the previous cell cycle acquires its appendages.

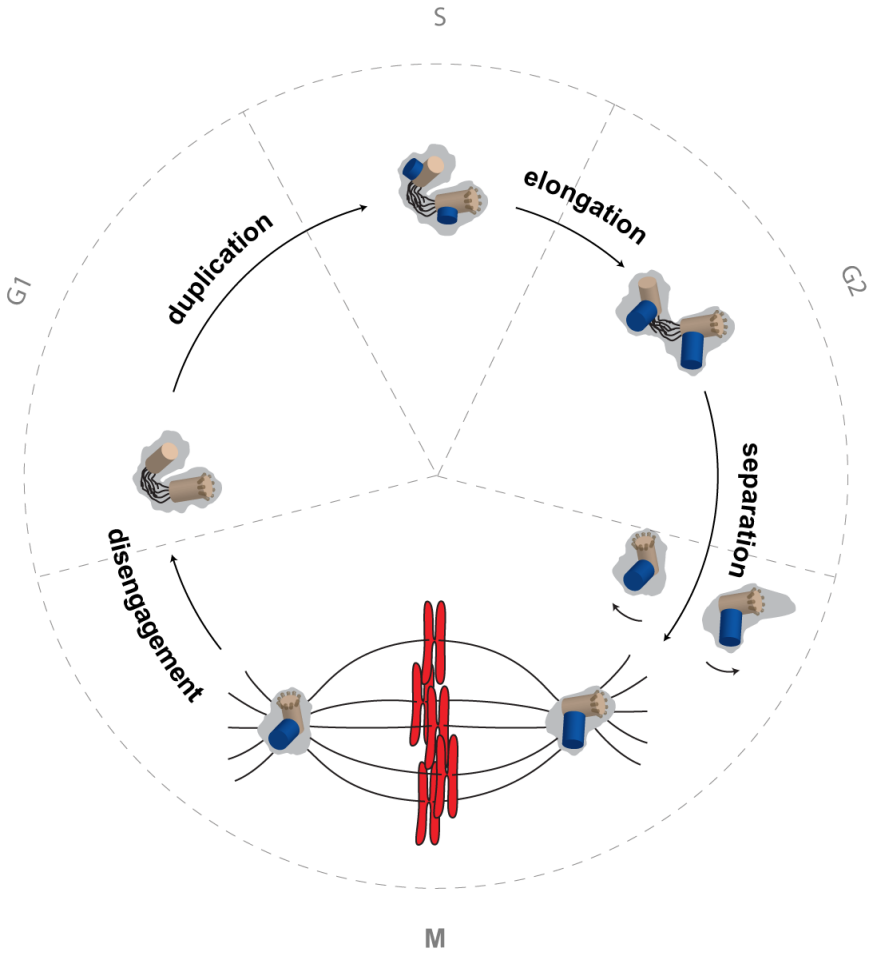

Figure 3. The centrosome cycle. Schematic illustration of the centrosome cycle in relation to the cell cycle. Mature centrioles are depicted in gray, procentrioles in dark blue, chromosomes in red. The two centrioles of a G1 phase cell duplicate upon entry into S phase and elongate to reach their final length during the following G2 phase. At the onset of mitosis, the centrosome is separated into two to organize the spindle poles of the mitotic spindle. Centriole disengagement at the exit from mitosis of the previously tightly connected centrioles prepares for the next round of duplication.

2.2.1 Centriole Biogenesis in *Caenorhabditis elegans*

Crucial insight into centriole biogenesis and specifically centriole duplication was gained through pioneering studies in *Caenorhabditis elegans*. This revealed that just five essential proteins are essential for procentriole assembly: the coiled-coil proteins SPD-2, SAS-4,

SAS-5 and SAS-6 and the protein kinase ZYG-1 (Figure 4; O'Connell *et al.,* 2001; Kirkham *et al.,* 2003; Leidel and Gonczy 2003; Delattre *et al.,* 2004; Leidel *et al.,* 2005; Delattre *et al.,* 2006; Pelletier *et al.,* 2006; Dammermann *et al.,* 2008). First, SPD-2 is recruited to the paternal centriole shortly after fertilization of the egg. This allows recruitment of ZYG-1, which in turn localizes a complex of SAS-5 and SAS-6 and initiates the formation of the "central tube" in close proximity to the pre-existing centriole. In this context, it has been proposed that ZYG-1-mediated phosphorylation of SAS-6 at Ser123 is necessary for central tube formation and maintenance of Sas-6 at the central tube (Kitagawa *et al.,* 2009). The SAS-5/SAS-6 complex then recruits SAS-4 to facilitate the assembly of MTs onto the central tube (Pelletier *et al.,* 2006).

Importantly, the overall pathway of centriole biogenesis is highly conserved from *C. elegans* to humans at both a morphological and molecular level. SPD-2, SAS-4 and SAS-6 have orthologues in human cells termed Cep192 (Andersen *et al.,* 2003), CPAP/CENPJ/hSas-4 (Hung *et al.,* 2000) and hSas-6 (Leidel *et al.,* 2005), respectively. Even though ZYG-1 does not have obvious structural orthologues in organisms outside nematodes, a functional analogue has been identified in Plk4 in *Drosophila* and human cells (Bettencourt-Dias *et al.,* 2005; Habedanck *et al.,* 2005). Interestingly, Plk4 does not seem to require Cep192 for recruitment to the centriole in human cells (Kleylein-Sohn *et al.,* 2007). Similar to ZYG-1, the search for a functional orthologue of SAS-5 has long remained unsuccessful. Yet recently, the *Drosophila* protein Ana2 and the human protein STIL have been suggested to be functional orthologues (Stevens *et al.,* 2010).

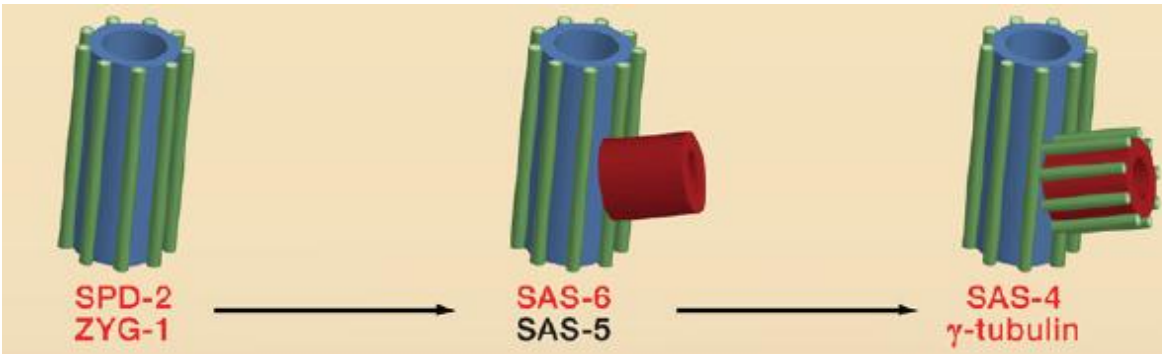

Figure 4. Centriole duplication in *Caenorhabditis elegans*. SPD-2 recruits the protein kinase ZYG-1, which then recruits a complex of SAS-5 and SAS-6. This promotes the formation of a central tube (red) onto which centriolar microtubules (green) are assembled by SAS-4. Proteins highlighted in red have functional orthologues in vertebrates (adapted from Nigg and Raff 2009).

2.2.2 Centriole Biogenesis in Human Cells

As described above, the core components of centriole biogenesis are well conserved from worm to man. Indeed, detailed studies have revealed that human procentriole assembly follows a very similar route as in *C. elegans* (Figure 5). Polo-like kinase 4 (Plk4) has been identified as the pivotal protein in centriole biogenesis in *Drosophila* and human cells (Bettencourt-Dias *et al.,* 2005; Habedanck *et al.,* 2005). Depletion of Plk4 inhibits centriole duplication and its overexpression induces centriole overduplication, identifying Plk4 as the key protein regulating "copy-number control" (reviewed in Nigg 2007; see also 2.2.3). This suggests that Plk4 protein levels must be tightly regulated in order to ensure correct centrosome number. A study performed in osteosarcoma (U2OS) cells which could be induced to overexpress active Plk4 was used to delineate the human centriole biogenesis pathway (Kleylein-Sohn *et al.,* 2007). Herein, excess Plk4 leads to the formation of multiple procentrioles in a rosette-like arrangement around the pre-existing centrioles. Accordingly, at the G1/S phase transition Plk4 sequentially recruits hSas-6, γ-tubulin, CPAP and Cep135 to the site of procentriolar outgrowth. HSas-6 is exclusively found at the nascent procentriole where it is required for the formation of the cartwheel which most likely confers the nine-fold symmetry (Nakazawa *et al.,* 2007). Even though the cartwheel is a constitutive component of *Drosophila* centrioles, it is restricted to the procentriole stage in vertebrates (Alvey 1986), the time when hSas-6 levels peak (Strnad *et al.,* 2007). In contrast, the cartwheel component Cep135 (Hiraki *et al.,* 2007) also remains associated with the centriole after completion of centriole duplication and the disappearance of the cartwheel (Kleylein-Sohn *et al.,* 2007). Centriole elongation is initiated after the recruitment of γ-tubulin which enables nucleation of centriolar microtubules. The growing procentriole is then decorated with CP110 which marks the distal tip of both nascent and mature centrioles. CPAP most likely serves to insert tubulin underneath the CP110 cap and thereby contributes to the control of centriole elongation (Kohlmaier *et al.,* 2009; Schmidt *et al.,* 2009b; Tang *et al.,* 2009). Interestingly, CPAP and CP110 have opposing functions in centriole elongation as overexpression of CPAP yields overly long centrioles and overexpression of CP110 suppresses this effect. Moreover, POC5, POC1 and OFD1 have also been shown to be involved in centriole length control (Azimzadeh *et al.,* 2009; Keller *et al.,* 2009; Singla *et al.,* 2010).

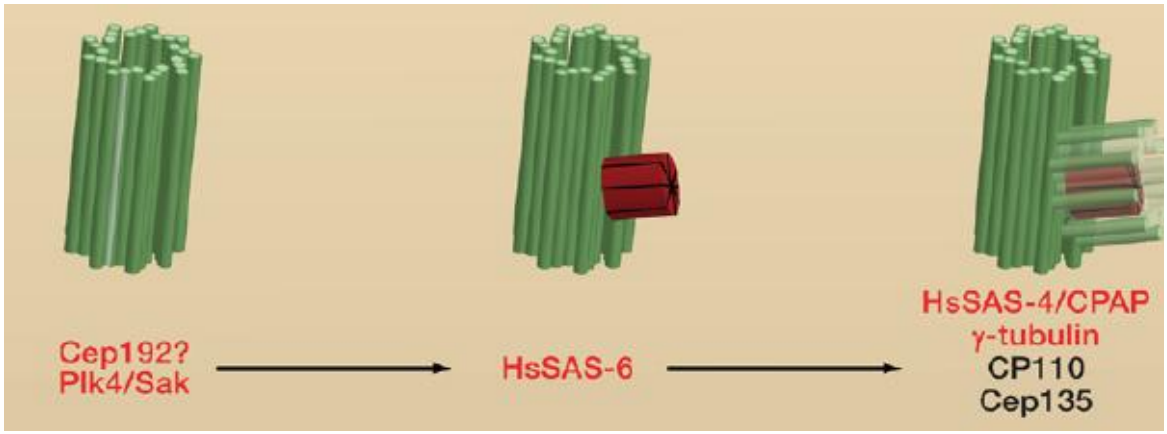

Figure 5. Centriole duplication in humans. Even though Cep192 is the human homologue of *C. elegans* SPD-2, it does not appear to be essential for centriole duplication. The functional orthologue of *C. elegans* ZYG-1, Plk4, recruits hSas-6 which seems to be required for the formation of a central cartwheel structure (red). CPAP and γ-tubulin are then required to convert this structure into a procentriole onto which CP110 and Cep135 are assembled. Proteins that have functional orthologues in *C. elegans* are depicted in red (adapted from Nigg and Raff 2009).

2.2.3 Regulation of Centriole Duplication

Aberrant centrosome numbers perturb bipolar spindle formation which is strictly required to ensure faithful chromosome segregation during mitosis. As cells do not have a checkpoint to sense abnormal centrosome numbers as for the completion of DNA-replication and MT-kinetochore attachment, other mechanisms have to guarantee proper centrosome numbers. This is achieved through precise control of centriole duplication by means of "cell-cycle control" and "copy-number control" (Figure 6).

2.2.3.1 Cell-Cycle Control

Temporal control of centriole duplication is achieved by synchronization of the centrosome cycle with the chromosome duplication cycle. Centriole duplication is only initiated during S phase and progression through the cell cycle is required to initiate a new round of centriole duplication (Balczon *et al.,* 1995; Meraldi *et al.,* 1999). The exception to this rule is only seen in certain cancer cell lines, e.g. U2OS and CHO cells (Kuriyama *et al.,* 1986; Balczon *et al.,* 1995). This mode of control is reminiscent of DNA replication, both in respect to the timing during the cell cycle and in the sense that a licensing step during the cell cycle prevents premature re-replication (Tsou and Stearns 2006a; Hook *et al.,* 2007). Here, the licensing step corresponds to the loading of the minichromosome maintenance (Mcm) 2-7 proteins to form the pre-replicative complex (preRC) during late mitosis and G1 when CDK activity is low. DNA replication is then initiated by high

CDK2 activity in the following S phase. Simultaneously, CDK activity prevents premature re-licensing until the completion of mitosis (reviewed in Diffley 2001; Blow and Dutta 2005).

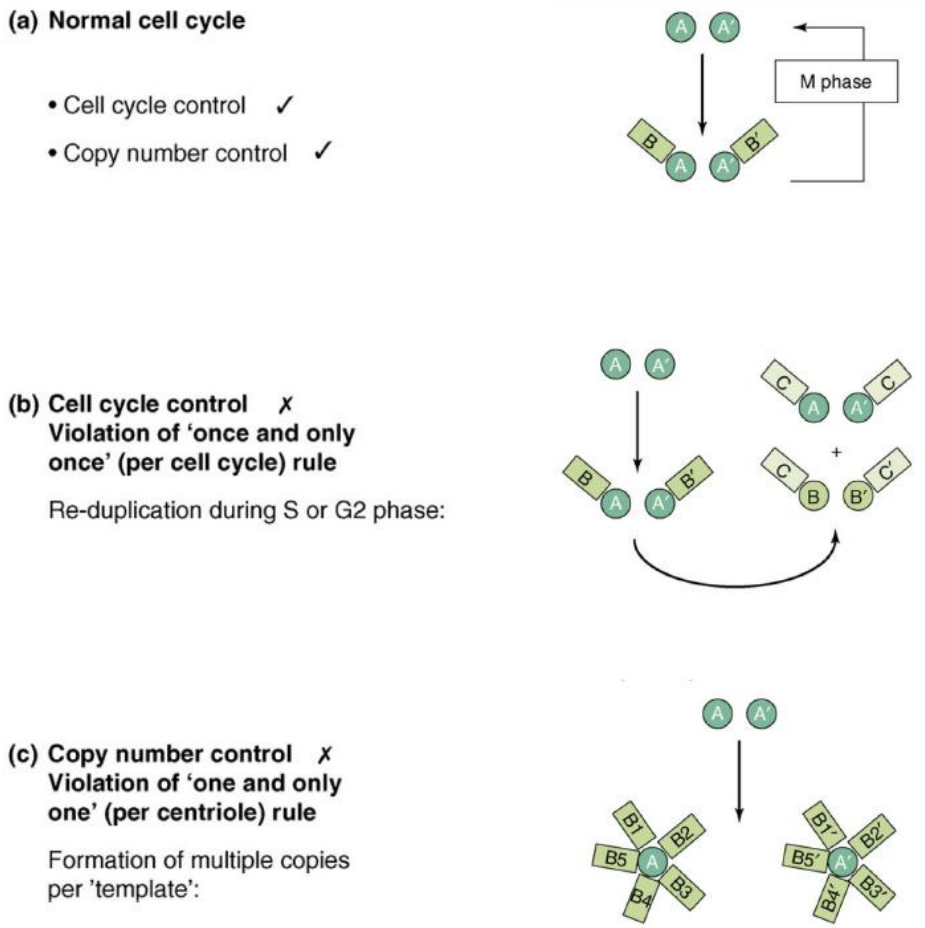

Figure 6. Control of centriole duplication. Cell cycle and copy number control govern the centrosome cycle. Violation of either rule leads to aberrations in centrosome numbers. **(a)** Centriole duplication in a normal cell cycle gives rise to two procentrioles (B and B´) from two parental centrioles (A and A´). **(b)** Cell cycle control ensures that a new round of duplication can only occur after passage through M phase. **(c)** Copy number control is exerted by Plk4 and ensures that only one procentriole is formed per pre-existing centriole (adapted from Nigg 2007).

Analogous to DNA replication, centriole duplication is also triggered by CDK2 activity at the beginning of S phase. Here, Cdk2/Cyclin-E is required for procentriole biogenesis (Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999; Matsumoto *et al.*, 1999) and Cdk2/Cyclin-A for re-duplication during prolonged S phase arrest in certain cancer cell lines (Meraldi *et al.*, 1999). In contrast, Cdk2 and Cyclin-E knockout mice show no obvious defects in centriole duplication (Berthet *et al.*, 2003; Geng *et al.*, 2003; Ortega *et al.*, 2003; Duensing *et al.*, 2006). It is conceivable that in these mice, other Cdks or

Cyclins compensate for the loss of Cdk2 or Cyclin-E because in mice lacking all interphase Cdks (Cdk2, Cdk3, Cdk4, Cdk6), Cdk1 associates with D-type and E-type cyclins to drive mitosis (Santamaria *et al.,* 2007).

The existence of a licensing mechanism inhibiting centriole re-duplication was first uncovered through cell fusion experiments in which disengaged, unduplicated G1 centrosomes were shown to duplicate in an S phase cytoplasm whereas engaged, duplicated G2 centrosomes did not (Wong and Stearns 2003). This suggested that the presence of an engaged procentriole inhibits centriole re-duplication. Laser ablation experiments supported this notion, as ablation of an engaged procentriole promoted re-duplication in S phase-arrested HeLa cells which ordinarily do not reduplicate in prolonged S phase (Loncarek *et al.,* 2008). Mechanistically, this intrinsic block to re-duplication has been proposed to be mediated by the control of centriole disengagement by the cysteine protease Separase in cooperation with Polo-like kinase 1 (Plk1) during late mitosis or early G1 to license centrioles for duplication in S phase (Tsou and Stearns 2006b; Tsou *et al.,* 2009). In this context the cysteine protease Separase might cleave a yet-to-be identified protein that tethers the two engaged centrioles, although this awaits direct demonstration. Separase is inhibited during S phase, G2 phase and the first part of mitosis before it is activated by the anaphase-promoting complex/cyclosome (APC/C) during the metaphase-anaphase transition. Hence, the aforementioned model fails to explain why certain cell types undergo centriole disengagement and centriole (re-)duplication in the absence of Separase activity. This is the case in *Drosophila* wing discs depleted of Cdk1 (Vidwans *et al.,* 2003), which is required for Separase activation, in S phase-arrested U2OS or CHO cells in which Separase should be inactivated by Securin (Kuriyama *et al.,* 1986; Balczon *et al.,* 1995; Dodson *et al.,* 2004) and even in S phase-arrested cells deficient of Separase (Tsou *et al.,* 2009). Moreover, multiple centrioles formed during ciliogenesis disengage during interphase before moving to the plasma membrane (Dirksen 1991).

2.2.3.2 Copy-Number Control

In addition to the cell-cycle control of centriole duplication which ensures that centrioles duplicate once and only once during each cell cycle, the cell also limits the number of procentrioles that are generated during each round of duplication. Canonical centriole duplication in dividing cells leads to the formation of one procentriole adjacent to one pre-

existing centriole. In contrast, hundreds of basal bodies form near-simultaneously in multi-ciliated epithelial cells.

The breakthrough in understanding the mechanism of copy-number control was made with the identification of Polo-like kinase 4 (Plk4) as the key regulator of this process in both humans (Habedanck *et al.*, 2005) and *Drosophila* (Bettencourt-Dias *et al.*, 2005), where Plk4 is known as Sak. This conclusion is justified by the fact that Plk4 protein levels directly correlate with centriole number. Lack of Plk4 inhibits centriole duplication and causes sequential loss of centrioles in successive cell divisions. Excess Plk4, on the other hand, triggers the simultaneous formation of supernumerary *bona fide* procentrioles which are arranged in a rosette-like manner around the parental centriole (Habedanck *et al.*, 2005; Kleylein-Sohn *et al.*, 2007). Excess Plk4 is furthermore capable of triggering *de novo* centriole formation in unfertilized *Drosophila* eggs (see also 2.2.3.3; Peel *et al.*, 2007; Rodrigues-Martins *et al.*, 2007a). Importantly, the triggering of procentriole formation absolutely requires Plk4 kinase activity (Habedanck *et al.*, 2005).

The formation of multiple procentrioles around the proximal end of the parental centriole argues that the maximum number of procentrioles might be dictated by spatial constraints instead of the availability of a pre-defined assembly site, as had been suggested previously (Jones and Winey 2006; Tsou and Stearns 2006a). In concordance with this model and the idea that parental centrioles constitute assembly platforms (Rodrigues-Martins *et al.*, 2007b), it would be plausible that Plk4 marks the assembly sites on the parental centriole cylinder by phosphorylation of yet-to-be identified substrates, which subsequently recruit the first procentriolar proteins, i.e. hSas-6, Cep135. This would thus form a "seed" for the nascent procentriole, which would subsequently be very rapidly expanded into nascent procentriolar structures. In line with this, excess hSas-6 also leads to the formation of supernumerary procentrioles (Leidel *et al.*, 2005; Peel *et al.*, 2007; Rodrigues-Martins *et al.*, 2007a; Strnad *et al.*, 2007). Thus, the number of centrioles formed during each S phase may be dictated by limiting of amounts of Plk4 that in turn recruit limiting amounts of hSas-6 to the parental centriole.

2.2.3.3 Canonical versus *de novo* Centriole Duplication

Most centrioles arise in the canonical, semi-conservative fashion at the proximal end of a parental centriole. However, centrioles can also form *de novo* in the absence of any pre-

existing centrioles. While the centrioles in most mammalian zygotes stem from the sperm, the first embryonic divisions in mouse zygotes are acentrosomal before each cell assembles the correct number of centrioles *de novo* during the blastomere stage. Afterwards, the centrioles are propagated via the canonical pathway (Szollosi *et al.,* 1972). Moreover, multiciliated cells can arise from overduplication of centrioles via *de novo* formation. In the latter case, hundreds of centrioles form around amorphous EM-dense granules composed of various centrosomal proteins which eventually fuse to form deuterosomes (Sorokin 1968). Interestingly, Plk4 seems to be highly expressed in these cells, at least in mice (Fode *et al.,* 1994), insinuating that increased Plk4 levels may be involved in the generation of multiciliated cells.

The canonical and *de novo* pathways rely on the same core mechanisms. Both require entry into S phase (Uetake *et al.,* 2007) and the same set of centriole duplication proteins, Plk4, hSas-6 and CPAP (Peel *et al.,* 2007; Rodrigues-Martins *et al.,* 2007a). Intriguingly, even though the presence of pre-existing centrioles inhibits *de novo* centriole formation, the *de novo* pathway can be induced in cycling, somatic vertebrate cells by removal of all resident centrioles (Khodjakov *et al.,* 2002; La Terra *et al.,* 2005; Uetake *et al.,* 2007). Importantly, the latter happens at the expense of numerical control of centriole number, even though levels of Plk4 and hSas-6 remain low.

2.3 Polo-like Kinase 4 (Plk4)

The Polo-like kinase family consists of four members: Plk1, Plk2 (Snk), Plk3 (Fnk) and Plk4 (Sak), of which Plk4 is the most divergent member. All four kinases share a structurally similar N-terminal kinase domain, which spans amino acids 12-265 in Plk4 (Figure 7). While Plks1-3 have two polo box motifs in common that, together, form a phosphopeptide binding domain which determines subcellular targeting and kinase regulation (Elia *et al.,* 2003a), Plk4 harbors only a single polo box motif at its C-terminus (Leung *et al.,* 2002). This indicates that Plk4 may not dock to substrates in the manner that is described for Plks1-3 (Lowery *et al.,* 2005).

Just N-terminal to Plk4's polo box lies the loosely defined, so-called cryptic polo box which acts as a dimerization domain (Leung *et al.,* 2002; Habedanck *et al.,* 2005) and is additionally required for centriolar localization (Habedanck *et al.,* 2005). Hence, in

contrast to Plk1 in which the two polo boxes form the phosphopeptide binding polo box domain (PBD; Cheng *et al.*, 2003; Elia *et al.*, 2003b); crystals of the single Plk4 polo box reveal intermolecular dimers (Leung *et al.*, 2002). Between the C-terminal single polo box of Plk4 and the N-terminal kinase domain lies an approximately 500 amino acid region, termed the linker region, which shares no similarity to other Plks and is not well conserved in *Drosophila* Plk4. Moreover, human Plk4 localizes to centrosomes in *Drosophila* cells but does not trigger centriole overduplication (Carvalho-Santos *et al.*, 2010). The same holds true for *Drosophila* Plk4 in human cells. This indicates that taxon-specific changes in regard to protein regulation and/or function have evolved.

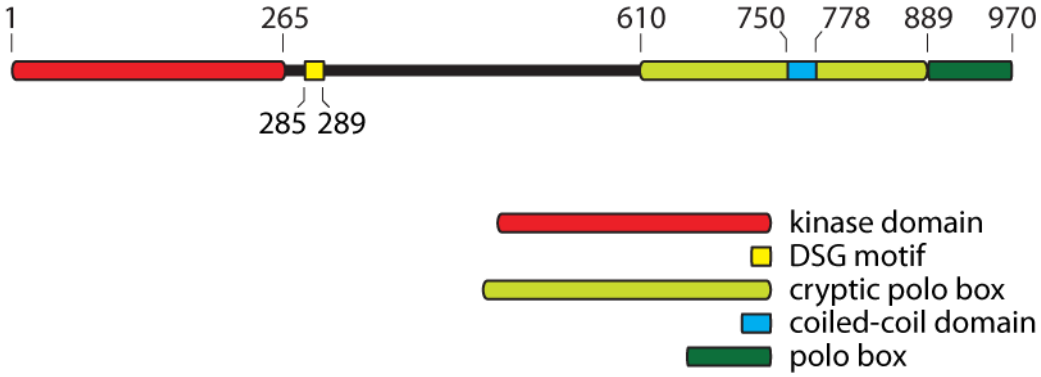

Figure 7. Domain structure of Plk4. Illustration of Plk4's functional domains. Schematic is drawn to scale.

Plk4 was first identified in mouse during a search for proteins regulating sialylation (Fode *et al.*, 1994) before the human homologue was separately identified in a PCR-based search for novel kinases involved in cancer development (Karn *et al.*, 1997). In humans, the *plk4* gene is located on chromosome 4 at locus 4q28 which has been implicated in frequent rearrangements and loss in tumor cells (Hammond *et al.*, 1999). Indeed, heterozygous $Plk4^{+/-}$ mice are prone to tumor development (Ko *et al.*, 2005). This may be due to the fact that $Plk4^{+/-}$ MEFs (mouse embryonic fibroblasts) display increased numbers of centrosomes and abnormal spindles. Yet, how Plk4 haploinsufficiency contributes to this phenotype remains unclear. $Plk4^{-/-}$ knockout mice, however, show a much more dramatic phenotype as they arrest in development shortly after gastrulation (Hudson *et al.*, 2001).

2.4 The Centrosome and Cancer

A direct link between centrosomal aberrations and cancer had already been proposed by Theodor Boveri in 1914 (Boveri 1914). He put forward the idea that deviations in centrosome numbers might contribute to the development of cancer through generation of multipolar spindles and erroneous mitosis. In recent years, Boveri's notion has been reawakened as centrosome aberrations are observed in many different cancers (Lingle *et al.,* 2002; Pihan *et al.,* 2003) and often accompanied with extensive chromosome aberrations (D'Assoro *et al.,* 2002; Pihan *et al.,* 2003), an indication of poor clinical outcome (Gisselsson 2003).

The accumulation of supernumerary centrosomes may occur via four different mechanisms (reviewed in Nigg and Raff 2009). First, genuine deregulation of the centrosome cycle may lead to excessive centriole duplication as has been described for human cells with excess Plk4 (Habedanck *et al.,* 2005), hSas-6 (Leidel *et al.,* 2005) or human papillomavirus E7 (Duensing *et al.,* 2000). Additionally, successive rounds of centriole duplication within the same S phase may also lead to supernumerary centrioles (Balczon *et al.,* 1995; Meraldi *et al.,* 1999). Second, cytokinesis failure or cell fusion can lead to tetraploid cells with four centrosomes. Third, fragmentation of the pericentriolar material may form extra spindle poles even though this does not represent true centrosome amplification. Finally, upregulation of PCM components may lead to the formation of additional procentrioles (Loncarek *et al.,* 2008; reviewed in Salisbury 2008).

In dividing cells each centrosome normally gives rise to one spindle pole and supernumerary centrosomes should result in multiple spindle poles and consequently in multipolar spindles. This is however not inescapably the case as cells have devised several mechanisms to form a bipolar spindle despite the presence of excess centrosomes (reviewed in Acilan and Saunders 2008; Godinho *et al.,* 2009). Centrosome inactivation, for instance, allows only two centrosomes to function as MTOCs during mitosis. Centrosome removal on the other hand, reduces the *de facto* number of centrosomes during gametogenesis. Alternatively, asymmetric segregation during cell division can also reduce the number of centrosomes so that one daughter cell inherits only one centrosome which it can then propagate during subsequent cell divisions. However, the predominant way for cancer cells to achieve bipolar mitoses is through clustering centrosomes into two spindle poles (Quintyne *et al.,* 2005; Saunders 2005; Basto *et al.,* 2008; Kwon *et al.,* 2008;

Yang *et al.,* 2008). Yet, cells undergoing centrosome clustering may nevertheless form merotelic kinetochore-MT attachements (one kinetochore attached to two spindle poles) which may aid the generation of chromosomal instability (Ganem *et al.,* 2009).

Considering that many tumors harbor centrosome abnormalities, clinical approaches to specifically target cells with extra centrosomes have been discussed as therapeutic approaches. This would exploit that cancer cells with extra centrosomes depend on certain proteins or pathways for their survival that are less critical in normal cells. Inhibition of these pathways would thus selectively kill cancer cells with extra centrosomes while leaving cells with normal centrosome numbers unharmed. In *Drosophila*, for example, the spindle assembly checkpoint (SAC) suddenly becomes essential in cells with excess centrosomes even though the SAC is not essential in normal *Drosophila* cells (Buffin *et al.,* 2007). Alternatively, human cancer cells with clustered supernumerary centrosomes but not cells with normal centrosome numbers are effectively killed by inhibition of centrosome clustering through perturbation of HSET function, a kinesin-related motor (Kwon *et al.,* 2008).

Despite evidence linking centrosome abnormalities and cancer, the lack of direct genetic proof hinders the establishment of a causal relationship (reviewed in Nigg and Raff 2009). This may be due to the fact that a large number of proteins is involved in centrosome assembly and that many of these genes may be mutated in cancer but the mutation frequency in any one particular gene is low.

2.5 The Ubiquitin-Proteasome System

The maintenance of genomic integrity relies on the faithful progression through the cell cycle which in turn is ensured by a network of phosphorylation and protein degradation events. Pivotal to protein degradation is the ubiquitin-proteasome system which catalyzes the proteolysis of proteins which are destined for degradation.

2.5.1 Ubiquitin-dependent Protein Degradation

A central component of the ubiquitin-proteasome system is the 76 amino acid small protein ubiquitin which is covalently attached via the glycine residue at its C-terminus to the ε-amino group of a lysine in the degradation target (reviewed in Hochstrasser 1996;

Hershko and Ciechanover 1998). This is carried out by the sequential action of one ubiquitin-activating enzyme (E1), one of several ubiquitin-conjugating enzymes (E2) and one of many ubiquitin ligases (E3) (Figure 8). First, the E1 enzyme adenylates ubiquitin to catalyze its covalent attachment to a cysteine in the active site of the E1 enzyme through a thioester bond. The activated ubiquitin moiety is then transferred onto a ubiquitin-conjugating enzyme in a *trans*-thiolation reaction which again entails the formation of a thioester bond with a cysteine in the active site of the E2 enzyme. Subsequently, the E2-ubiquitin complex is incorporated into the ubiquitin ligase. This multi-subunit protein complex then coordinates the E2-ubiquitin complex and the ubiquitination substrate to enable ubiquitin transfer or, alternatively, it actively catalyzes the ubiquitin transfer itself. After the isopeptide bond linkage of ubiquitin to the substrate protein, a polyubiqutin chain is usually formed, in which the C-terminus of each ubiquitin unit is linked to a specific lysine residue, commonly Lys[48], of the previous ubiquitin. Polyubiquitinated proteins are then specifically recognized and degraded by the 26S proteasome in an ATP-dependent process (reviewed in Pickart and Cohen 2004; Finley 2009).

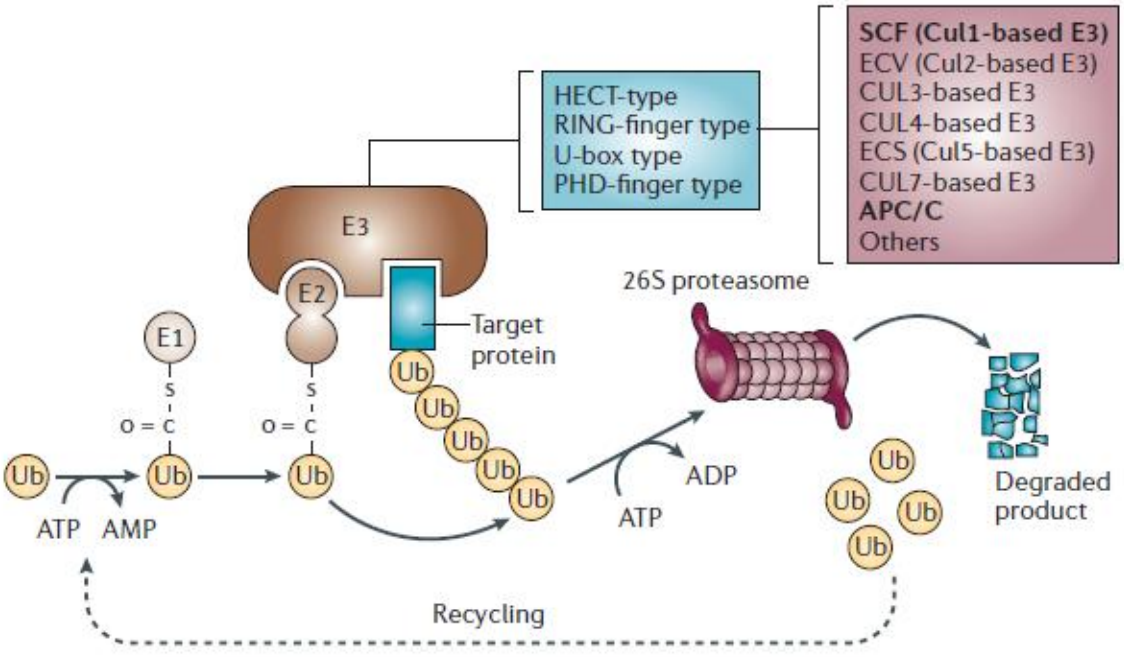

Figure 8. Overview of the ubiquitin-proteasome pathway. Ubiquitin (Ub) is activated in an ATP-dependent manner by the ubiquitin-activating enzyme (E1). The activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme (E2) which covalently attaches it to the target protein together with a multi-protein ubiquitin ligase (E3). The ubiquitinated protein is subsequently degraded by the 26S proteasome in an ATP-dependent manner. The four major classes of E3 ligases are depicted in blue and its largest subfamily, the RING-finger type Cullin-based E3s are depicted in red (adapted from Nakayama and Nakayama 2006)

2.5.2 The $SCF^{\beta TrCP}$ Complex

To achieve high substrate specificity, cells express many different E2 enzymes (about 30) and even more E3 ligases (more than 300). The latter are categorized into four major classes according to the presence of particular structural motifs: HECT-, RING-finger, U-box and PHD-finger-type E3 ligases (reviewed in Nakayama and Nakayama 2006). RING-finger-type E3 ligases comprise the largest group and are further subdivided into subfamilies. Among these, cullin-based E3 ligases are the largest single class of E3s.

2.5.2.1 Structure of SCF complexes

Cullin-based E3s are generally composed of a RING-finger protein, a scaffold protein, an adaptor protein and a receptor protein which confers the substrate specificity. In the case of the Skp1-Cul1-F-box protein (SCF) complex, the scaffolding function is provided by Cul1 which forms a core complex with the RING-finger protein Rbx1 and the adaptor protein Skp1 (Figure 9). Rbx1 binds the E2-ubiquitin complex, while Skp1 binds the F-box protein via its so-called N-terminal F-box named after its discovery in Cyclin F (Bai *et al.,* 1996). The F-box moiety of the SCF complex dictates its substrate specificity by recruiting substrate proteins through protein-protein interaction domains in its C-terminus. The substrate binding regions are also the basis for the classification of F-box proteins into three categories, namely, with WD40 repeats (FBXW), leucine-rich repeats (FBXL) or other domains (FBXO). Of the F-box proteins, three are thought to be involved in cell cycle control: SKP2 (FBXL1), FBW7 (FBXW7) and β-transducin repeat-containing protein (βTrCP). The latter exists in two biochemically indistinguishable paralogues: βTrCP1 (FBXW1) and βTrCP2 (FBXW11) (reviewed in Nakayama and Nakayama 2006). The term βTrCP will therefore be used to refer to both.

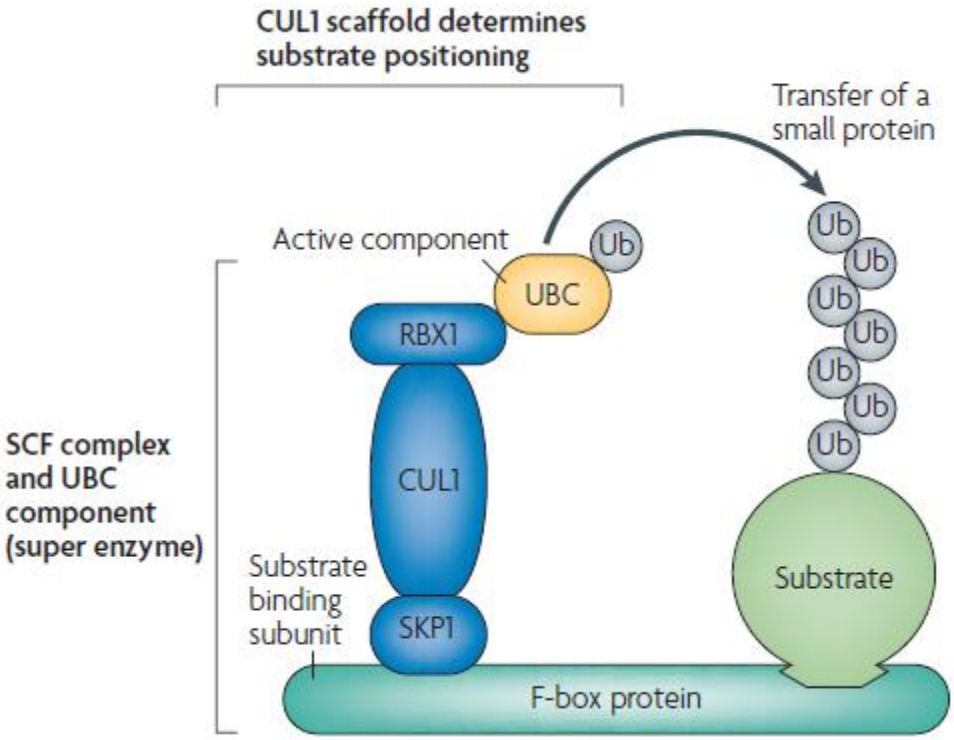

Figure 9. Structure of the SCF$^{\beta TrCP}$ complex. The SCF subunit Cul1 functions as a molecular scaffold and connects the adaptor subunit Skp1 and Rbx1. Skp1 binds the F-box protein βTrCP which recognizes the ubiquitination substrate while Rbx1 recruits the ubiquitin conjugating enzyme E2 (Ubc) (adapted from Frescas and Pagano 2008).

2.5.2.2 Regulation of βTrCP-mediated Degradation

The irreversibility of protein degradation demands accurate control over which protein is to be degraded at what point during the cell cycle, as premature or tardy protein degradation has detrimental effects for the cell (reviewed in Nakayama and Nakayama 2006). In regard to substrate recognition there is a clear conceptual difference between SCF-type and other ubiquitin ligases, namely activation of the ligase (anaphase promoting complex/cyclosome, APC/C) versus activation of the substrate (SCF; Reed 2003). In the case of APC/C, the ubiquitin ligase is activated through phosphorylation and the availability of co-factors. Once activated, APC/C readily recognizes its substrates through constitutive degrons, i.e. KEN-box or D-box. In contrast, SCF-type ligases require prior "activation" of their substrates. In most cases this activation occurs via phosphorylation of a degron motif in the substrate and the SCF complex then binds this phosphodegron via its F-box protein (Skowyra *et al.,* 1997). This allows versatile regulation of substrate recognition as degron phosphorylation itself is subject to both temporal and spatial regulation.

βTrCP recognizes a DSGxx[S/T] motif or derivates thereof ([D/E] instead of [S/T]) in its substrates (reviewed in Frescas and Pagano 2008). This oftentimes involves the recruitment of phosphodegron-directed kinases through phosphorylation-dependent docking sites. For instance, the Cdk1-inhibitory kinase Wee1 is first phosphorylated by Cdk1 which allows docking and phosphorylation of the DSG motif by Plk1 (Watanabe *et al.,* 2004). Other examples which follow a similar two-step mechanism, albeit not necessarily carried out by the same kinases, are the regulation of the cell cycle regulators β-catenin (Liu *et al.,* 2002) and Erp1 (Liu and Maller 2005; Rauh *et al.,* 2005; Hansen *et al.,* 2006).

2.5.2.3 The $SCF^{\beta TrCP}$ Complex at the Centrosome

A role for the $SCF^{\beta TrCP}$ complex in centrosome function has been implied by a multitude of evidence. The two structural components of the SCF complex, Skp1 and Cul1, have both been shown to localize to the PCM as well as to the centrioles (Freed *et al.,* 1999). Clues for a functional role of the $SCF^{\beta TrCP}$ complex at the centrosome came from the identification of the *Drosophila* homologues of βTrCP and Skp1 (Slimb and SkpA, respectively) as negative regulators of centriole duplication (Wojcik *et al.,* 2000; Murphy 2003). Mutation of either protein promoted centrosome amplification. Similarly, the analysis of $\beta TrCP^{-/-}$ null mice revealed a function of βTrCP in centrosome duplication as these mice exhibited supernumerary centrosomes (Guardavaccaro *et al.,* 2003). Further support for a role of proteasomal degradation in centriole duplication came from studies in U2OS cells which had been treated with the proteasome inhibitor Z-L_3VS (Duensing *et al.,* 2007). Proteasomal inhibition by this inhibitor lead to Plk4-dependent centriole overduplication in rosette-like arrangement, reminiscent of Plk4 (Habedanck *et al.,* 2005; Kleylein-Sohn *et al.,* 2007) and hSas-6 (Strnad *et al.,* 2007) overexpression.

2.5.2.4 Regulation of Plk4 Expression

The above-described phenotypes insinuate that Plk4 expression has to be tightly regulated for faithful centriole duplication. At transcript level, *plk4* expression is cell cycle regulated and mRNA levels are low in G_o and G1 phase and then increase from late G1 until they plateau in M phase (Fode *et al.,* 1996). Interestingly, *plk4* transcripts are elevated in colorectal cancer (Macmillan *et al.,* 2001). Active regulation of Plk4 protein levels had been suggested to depend on the presence of PEST motifs within Plk4

(Yamashita *et al.,* 2001) and lead to a short half-life of approximately 2-3 hours (Fode *et al.,* 1994). Insight into the regulation of Plk4 stability was recently gained by two studies in *Drosophila* which revealed that Plk4 harbors a conserved DSGxxT motif which regulates its SCF^{Slimb}-dependent degradation (Cunha-Ferreira *et al.,* 2009; Rogers *et al.,* 2009). Inactivation of Slimb led to increased Plk4 protein levels and concomitant centriole overduplication in the typical rosette-like arrangement of procentrioles around the parental centriole. Furthermore, a direct biochemical interaction between Slimb and Plk4 was demonstrated to depend on the double phosphorylation of the conserved DSG motif in Plk4. These works therefore described how adequate Plk4 protein levels are guaranteed in order to maintain correct centrosome numbers in *Drosophila*.

The revelation that Plk4 protein levels are regulated by βTrCP-mediated degradation not only gave insight into how fidelity of centriole duplication is ensured but also opened the door to new questions. It will be important to clarify whether this control of Plk4 protein levels is conserved from *Drosophila* to man and which kinases control the Plk4-βTrCP interaction through phosphorylation.

3 AIM OF THIS PROJECT

Plk4 had previously been demonstrated to be pivotal to centriole duplication as its kinase activity seems to be required to initiate centriole duplication and its protein levels directly correlate with centriole numbers. Yet, how Plk4 protein levels are regulated had not been resolved. This study aimed at uncovering how Plk4 protein levels are regulated to ensure faithful centriole duplication. First, we addressed whether Plk4 kinase activity is essential for centriole overduplication. Second, after the realization that βTrCP is responsible for targeting Plk4 for ubiquitination and degradation, we examined how Plk4 kinase activity contributes to controlling its βTrCP-mediated degradation. Finally, we addressed whether Plk4 kinase activity is sufficient for Plk4 degradation.

4 RESULTS

At the beginning of this work Plk4 had been recognized as a key protein in regulating centriole duplication (Bettencourt-Dias *et al.,* 2005; Habedanck *et al.,* 2005). It was known that Plk4 kinase activity is required to trigger the ordered integration of the centriole duplication proteins, hSas-6, CPAP, Cep135 and CP110 into the procentriole. Furthermore, Plk4 protein levels had been shown to directly correlate with centriole numbers. Plk4 had accordingly been termed to be the fundamental regulator of centriole copy number control (Nigg 2007). In spite of this, the mechanisms responsible for fine-tuning Plk4 protein levels to ensure precise centriole regulation were unidentified.

During the course of this study, antibodies were first generated to address the regulation of Plk4 protein levels. Then, we investigated how excess kinase-dead Plk4 triggers centriole overduplication. Encouraged by the possibility that kinase-dead Plk4 protects endogenous Plk4 from degradation, we explored if Plk4 protein levels are directly regulated by the $SCF^{\beta TrCP}$ complex before examining how Plk4 protein levels are regulated by the $SCF^{\beta TrCP}$ complex. Finally, we investigated whether Plk4 autophosphorylation is sufficient for βTrCP binding and undertook measures to identify a possible second kinase involved in regulating βTrCP-mediated degradation of Plk4.

4.1 Generation and Characterization of anti-Plk4 Antibodies

In order to complement the existing polyclonal rabbit anti-Plk4 antibodies, monoclonal anti-Plk4 antibodies were raised (kindly performed by A. Baskaya, C. Szalma and A. Uldschmid). To this end, mice were injected with purified, recombinant MPB-tagged Plk4 spanning amino acids 715-970. After an immune response had been monitored, mouse spleen cells were fused to myeloma cells and hybridoma cell clones were selected. Of these, two positive clones (93-80-4 and 93-302-11) were analyzed in more detail. Specificity of the monoclonal anti-Plk4 antibodies from both clones was confirmed by immunofluorescence of U2OS cells which had been depleted of Plk4 for 48 hours by siRNA oligonucleotides transfection (Figure 10A). Note that siRNA-mediated depletion of Plk4 leads to centriole loss over successive cell divisions, as visualized by anti-CP110 staining (see also Habedanck *et al.,* 2005). Both monoclonal anti-Plk4 antibodies also

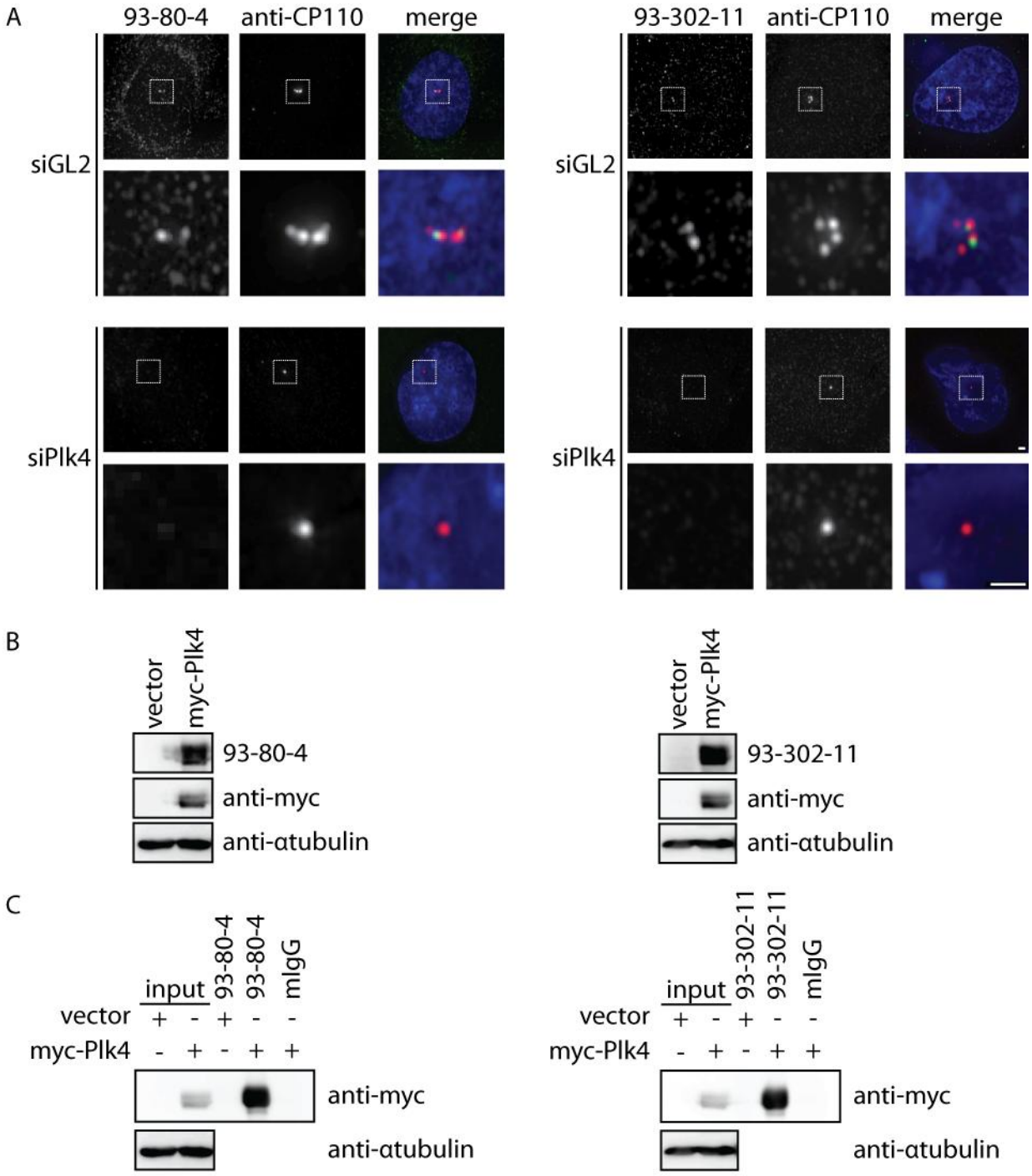

Figure 10. Characterization of two monoclonal anti-Plk4 antibodies. (A) U2OS cells were transfected for 48 hours with siRNA oligonucleotides targeting *GL2* or *Plk4*. Cells were fixed and stained with monoclonal anti-Plk4 antibodies (green): 93-80-4 (left panel) or 93-302-11 (right panel), anti-CP110 antibodies (red) and DAPI (blue). Magnifications of boxed areas are shown below the respective panels. Scalebar: 1 µm. **(B)** HEK 293T cells were transfected for 24 hours with myc-Plk4, lysed and the cell extracts were immunoblotted with anti-Plk4 antibodies: 93-80-4 (left panel) or 93-302-11 (right panel), anti-myc antibodies and anti-αtubulin antibodies. **(C)** myc-Plk4 was expressed in HEK 293T cells for 24 hours and the cell extracts were subjected to anti-Plk4 immunoprecipitations: 93-80-4 (left panel) or 93-302-11 (right panel). The precipitated proteins were analyzed by immunoblotting for the myc-epitope and αtubulin.

detected overexpressed myc-Plk4 by immunoblotting (Figure 10B) and in cell extracts by immunoprecipitations (Figure 10C). Yet, neither antibody detected endogenous Plk4 by immunoblotting which goes in line with the low abundance of endogenous Plk4 (data not shown; see also Bettencourt-Dias *et al.*, 2005; Habedanck *et al.*, 2005). To alleviate this, all experiments in this study requiring the detection of Plk4 via immunoblotting were carried out using overexpressed Plk4.

4.2 Kinase-dead Plk4 Causes Centriole Overduplication

The bottleneck of investigating Plk4's function in the regulation of centriole duplication has thus far been its low abundance. As a consequence, detection of endogenous human Plk4 or its *Drosophila* homolog, Sak, has remained impossible by means of immunoblotting (data not shown; see also Bettencourt-Dias *et al.*, 2005; Habedanck *et al.*, 2005). In order to be able to study Plk4 despite this drawback, transgenic U2OS T-REx cell lines that stably harbor the cDNA of human myc-tagged wild-type (U2OS:myc-Plk4-WT) or kinase-dead Plk4 (U2OS:myc-Plk4-KD) under control of a tetracycline-inducible CMV promoter were generated in our laboratory by Jens Westendorf.

4.2.1 Plk4-WT and Plk4-KD Trigger Centriole Overduplication

In concordance with previous results (Kleylein-Sohn *et al.*, 2007), 16 hours after induction of wild-type Plk4 expression in S phase-arrested U2OS:myc-Plk4-WT cells, approximately 80% of cells exhibited centrosomal myc-Plk4 localization and a rosette-like pattern of procentrioles around the pre-existing centrioles as revealed by CP110 staining (Figure 11A), which have previously been reported to be *bona fide* procentrioles (Habedanck *et al.*, 2005; Kleylein-Sohn *et al.*, 2007). Note, that staining for the proximal centriolar protein Cep135 does not allow visualization of engaged procentrioles (Figure 11A). The distal centriolar protein CP110 (Kleylein-Sohn *et al.*, 2007) has hence been used to identify procentrioles at early stages of centriole duplication during the remainder of this study.

Centriole overduplication has been demonstrated to depend on Plk4 kinase activity in transient overexpression experiments in different cell lines (Habedanck *et al.*, 2005; Sillibourne *et al.*, 2009). Yet, when we compared the ability of wild-type Plk4 (Figure

11A) and kinase-dead Plk4 (Figure 11B) to induce centriole overduplication in the U2OS:myc-Plk4 cell lines, we surprisingly observed robust centriole overduplication in both cell lines. Intriguingly, myc-Plk4-WT and myc-Plk4-KD induced a similar extent of centriole overduplication, which was indistinguishable by CP110 staining.

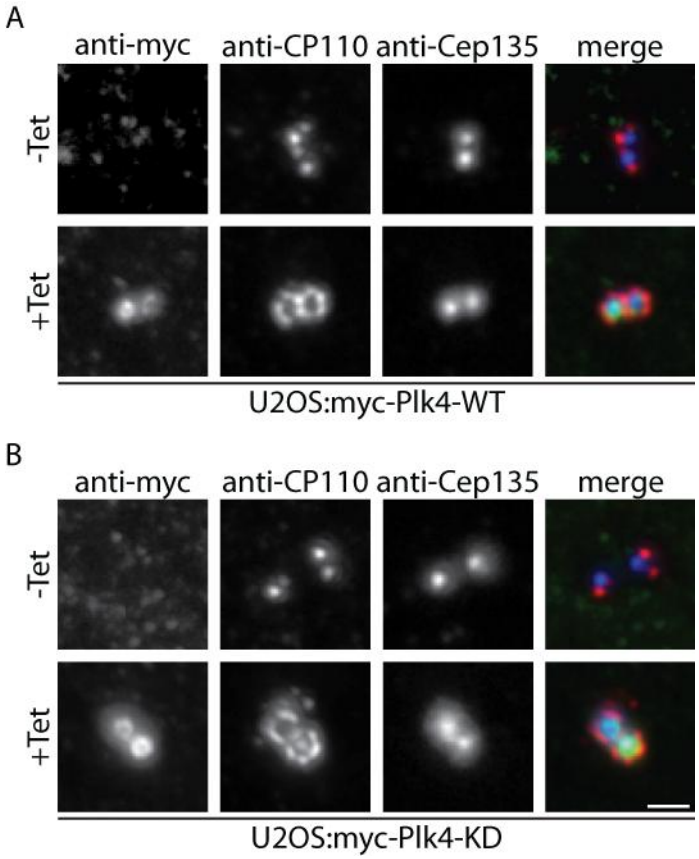

Figure 11. Excess Plk4 causes centriole overduplication. U2OS:myc-Plk4-WT or U2OS:myc-Plk4-KD cells were arrested with aphidicolin for 24 hours before expression of myc-Plk4-WT or myc-Plk4-KD was induced for 16 hours. No tetracycline was added to controls. Cells were fixed and stained with antibodies against the myc-epitope (green), CP110 (red) and Cep135 (blue). Scale bar: 1 µm**.**

Transient overexpression of kinase-dead Plk4 had also been observed previously to trigger centriole overduplication, albeit at very low levels (Habedanck *et al.*, 2005). At the time this had been attributed to cell division failure as centriole overduplication induced by kinase-dead Plk4 could be inhibited by blocking cell cycle progression (Habedanck *et al.*, 2005). Yet, as shown above, robust centriole overduplication occurred in S phase-arrested U2OS:myc-Plk4-KD cells (Figure 11B). This prompted us to investigate centriole overduplication in response to transient kinase-dead Plk4 overexpression more scrutinously, utilizing the distal centriolar protein CP110 as marker. This revealed that transient kinase-dead Plk4 overexpression was sufficient to induce centriole overduplication in the distinct rosette-like configuration of procentrioles around the older

centriole (Figure 12), which represents the hallmark of *bona fide* centriole overduplication and not the consequence of cytokinesis failure as suggested by Habedanck *et al*.

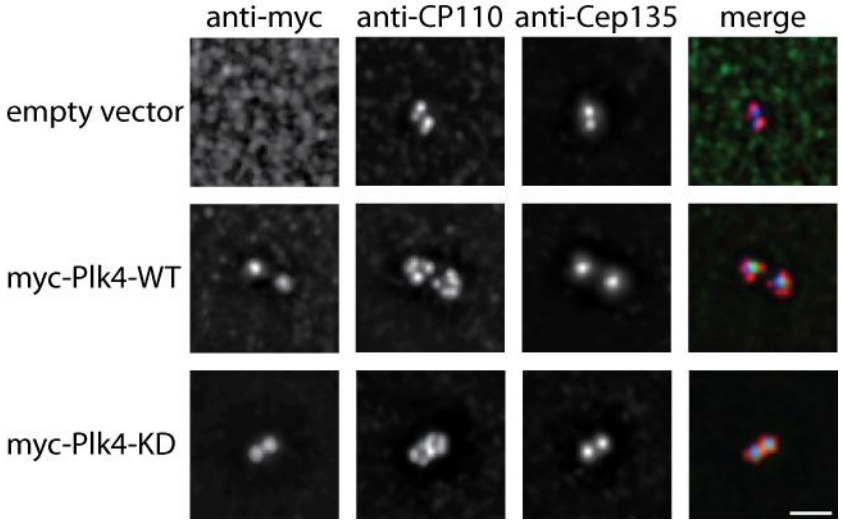

Figure 12. Transient kinase-dead Plk4 overexpression triggers *bona fide* centriole overduplication. U2OS cells were transfected for 48 hours with empty vector, myc-Plk4-WT or myc-Plk4-KD. Cells were fixed and stained with antibodies for the myc-eptitope (green), CP110 (red) and Cep135 (blue). Scalebar: 1 µm.

4.2.2 Endogenous Plk4 is Required for Plk4-KD-induced Centriole Overduplication

The surprising results that both transient and stable overexpression of kinase-dead Plk4 triggers centriole overduplication prompted us to investigate centriole overduplication more closely in the U2OS:myc-Plk4-KD cell line. RT-PCR experiments were performed to reveal that the cell line indeed harbored the D154A mutation (data not shown), which

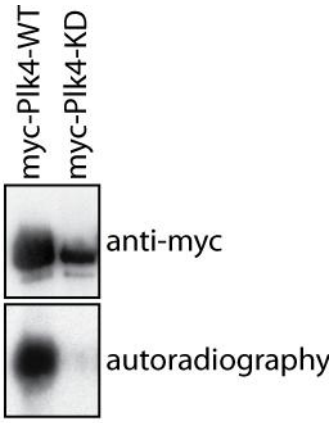

Figure 13. The D154A mutation renders Plk4 kinase dead. HEK 293T cells were transfected with myc-Plk4-WT or myc-Plk4-KD. The overexpressed proteins were immunoprecipitated with anti-myc antibodies and subjected to a kinase assay in the presence of γ-[^{32}P]-ATP. The kinase assay was analyzed by immunoblotting (upper panel) and autoradiography (lower panel). Myc-Plk4-KD carries an aspartate-to-alanine substitution at position 154.

abrogates Plk4 kinase activity (Figure 13).

Next, we carried out siRNA rescue experiments to determine whether the centriole overduplication phenotype upon kinase-dead Plk4 overexpression depends on endogenous wild-type Plk4. U2OS:myc-Plk4-WT and U2OS:myc-Plk4-KD cells were transfected for 24 hours with siRNA oligonucleotides targeting the 3´-untranslated region of Plk4 (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 (Figure 14A). Likewise, 80% of cells overexpressing myc-Plk4-WT still exhibited centriole overduplication even after depletion of endogenous Plk4. In stark contrast, centriole overduplication was reduced to 14% of cells upon expression of myc-Plk4-KD concomitant with transfection of siPlk4 3´-UTR (Figure 14B). A similar reduction of centriole overduplication was observed when either myc-Plk4-WT or myc-Plk4-KD were overexpressed in cells lacking hSas-6, 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.

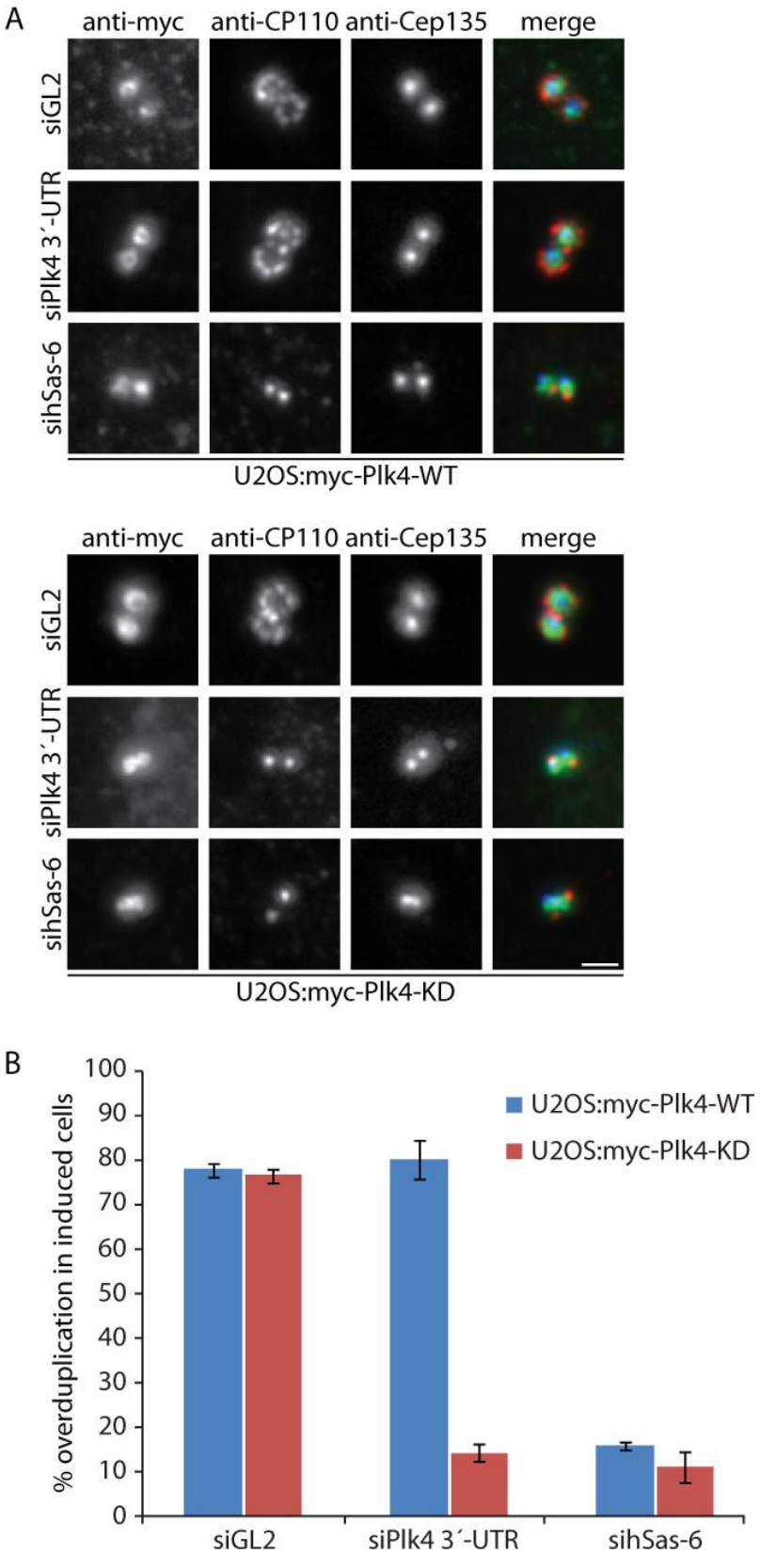

Figure 14. Centriole overduplication depends on endogenous Plk4. (A) U2OS:myc-Plk4-WT (upper panel) or U2OS:myc-Plk4-KD (lower panel) cells were transfected for 24 hours with siRNA oligonucleotides targeting *GL2*, the 3´-UTR of *Plk4* or *hSas-6* prior to induction of Plk4 expression (myc-Plk4-WT or myc-Plk4-KD) for 16 hours. Cells were stained against the myc-epitope (green), CP110 (red) and Cep135 (blue). Scale bar: 1 µm. **(B)** Percentage of cells treated as described in (A), which exhibit centriole overduplication. Data from three independent experiments (n = 100) are shown. Error bars denote s.e.m.

4.3 βTrCP-dependent Degradation of Plk4

Recent studies have shown that the levels of *Drosophila* Plk4/Sak are regulated by the ubiquitin-proteasome-pathway through the E3 ubiquitin ligase $SCF^{Slimb/\beta TrCP}$ (SKP1-CUL1-F-box-protein) (Cunha-Ferreira *et al.*, 2009; Rogers *et al.*, 2009). This elegantly demonstrated how cells regulate Plk4 kinase activity in order to prevent centriole overduplication. Even though the basic mechanism of this regulatory pathway had been uncovered, several questions regarding the control of Plk4 protein levels remained to be answered. First, is this pathway conserved from *Drosophila* to man? Second, how is this pathway regulated to allow controlled degradation of Plk4?

4.3.1 Centrosomal Plk4 Protein Levels are Regulated by the Proteasome

Previous work has revealed that proteasome inhibition leads to centriole overduplication in U2OS cells (Duensing *et al.*, 2007) and that protein levels of the *Drosophila* homolog of Plk4, Sak, are regulated in a Slimb/βTrCP-dependent manner. Together, this indicates that human Plk4 protein levels may also be regulated in a proteasome-dependent manner. Treatment of U2OS cells with low doses of MG132 for 16 hours led to centriole overduplication as described by Duensing *et al*., concomitant with increased Plk4 protein levels at the centrosome (Figure 15). This indicates that centrosomal Plk4 protein levels are regulated by the proteasome and deregulation of this pathway leads to centriole overduplication.

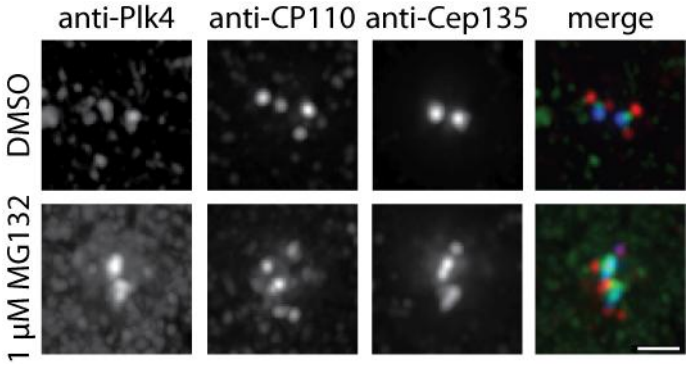

Figure 15. MG132 treatment increases centrosomal Plk4 levels and triggers centriole overduplication. U2OS cells were treated with DMSO or 1 µM MG132 for 16 hours, fixed and stained with anti-Plk4 (green), anti-CP110 (red) and anti-Cep135 (blue) antibodies. Scalebar: 1µm.

4.3.2 βTrCP is Required for Control of Plk4 Protein Levels and Centriole Number

After having shown that human Plk4 protein levels are regulated by the proteasome, we next investigated whether human Plk4 protein levels are also controlled by βTrCP as in *Drosophila*. To this end, 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 seven-fold compared to control cells (Figure 16A,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. While 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 (Figure 16A,C). Instead, these latter treatments increased the proportion of cells with fewer than 2 centrioles to 67% and 73%, respectively (Figure 16C). Hence, βTrCP is clearly required for the maintenance of correct centriole numbers and this in turn requires Plk4.

To demonstrate that βTrCP modulates overall Plk4 protein levels, we depleted βTrCP for 72 hours before inducing expression of myc-Plk4-WT for the last 24 hours of siRNA treatment followed by immunoblot analysis. Compared to cells treated with control siRNA duplexes (GL2), depletion of βTrCP led to a 1.5-fold increase in Plk4-WT protein (Figure 17A). Plk4 siRNA treatment carried out as control abolished Plk4 expression, as expected (Figure 17A). Conversely, co-expression of βTrCP and Plk4-WT in HEK 293T cells led to a decrease in Plk4 protein (Figure 17B). Together, the above data demonstrate that βTrCP is involved in modulating Plk4 protein levels in human cells and thus contributes to the maintenance of correct centriole number. This confirms and extends earlier work in *Drosophila* (Cunha-Ferreira *et al.*, 2009; Rogers *et al.*, 2009) and shows that the βTrCP-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 βTrCP was not emphasized (Korzeniewski *et al.*, 2009).

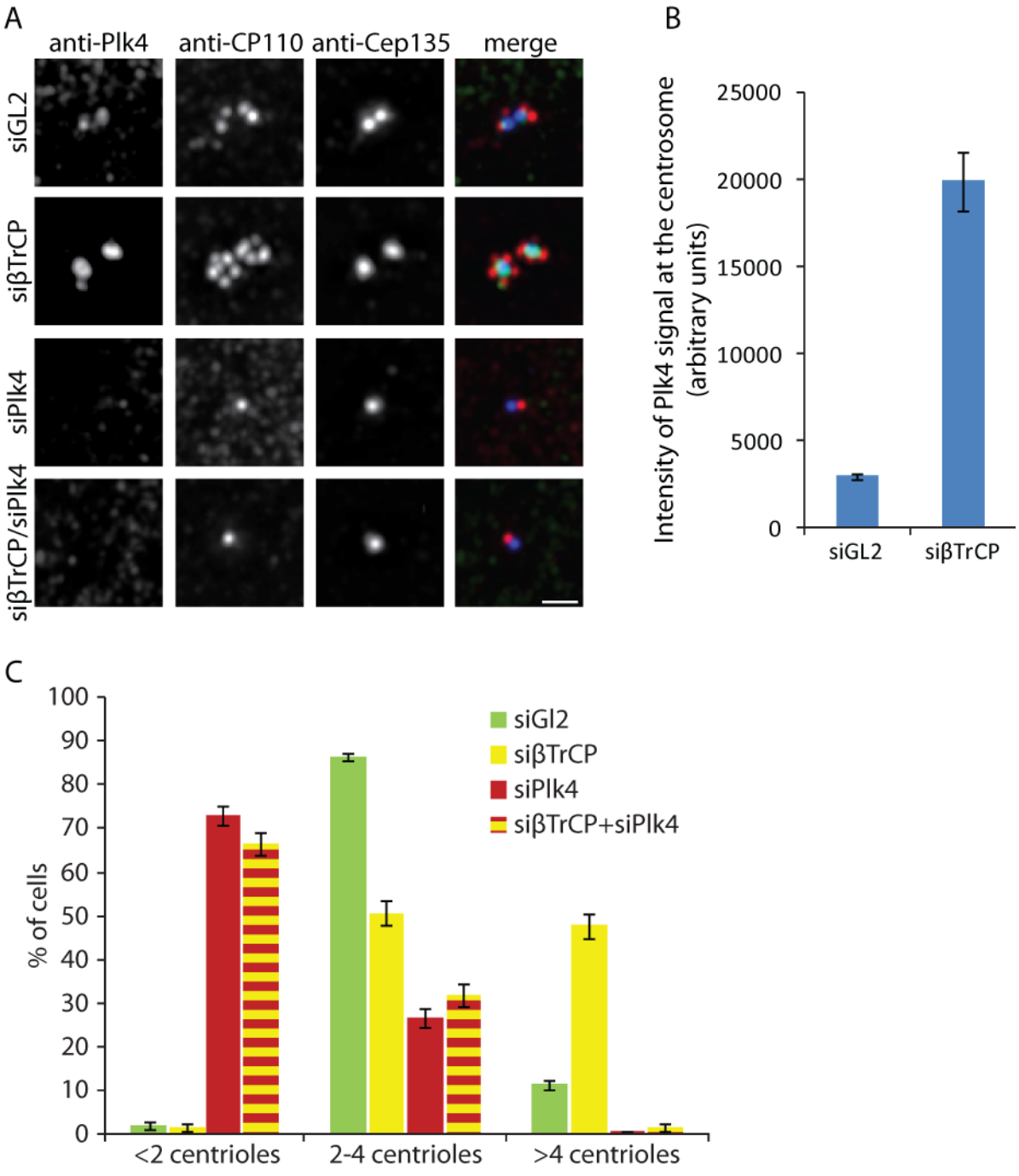

Figure 16. Plk4 protein levels and centriole number are controlled by βTrCP. (A) U2OS cells were transfected for 72 hours with siRNA oligonucleotides targeting *GL2*, *βTrCP*, *Plk4* or *βTrCP* and *Plk4* before cells were stained against Plk4 (green), CP110 (red) and Cep135 (blue). Scale bar: 1 µm. **(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) and grouped by the number of centrioles counted via CP110 staining. Data of three independent experiments (n = 100) are shown. Error bars denote s.e.m.

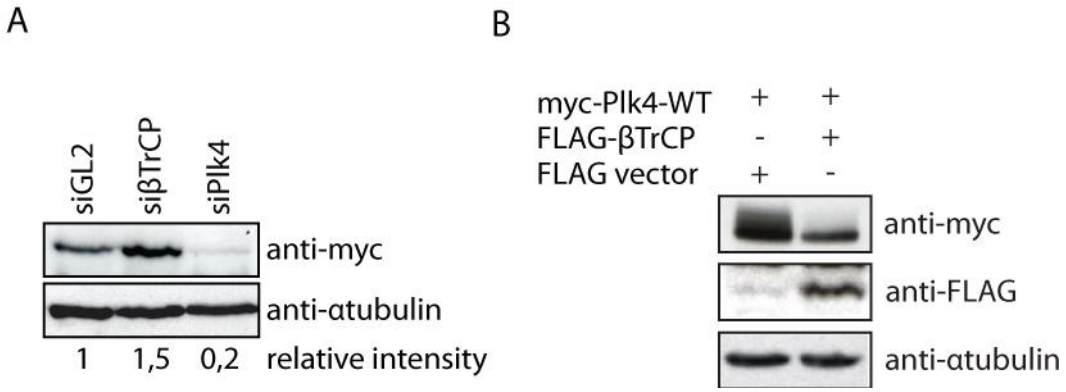

Figure 17. βTrCP controls overall Plk4 protein levels. (A) U2OS:myc-Plk4-WT cells were transfected for 72 hours with siRNA oligonucleotides targeting *GL2*, *βTrCP* or *Plk4*. The myc-signal was normalized against the αtubulin signal and quantified with ImageJ. Myc-Plk4-WT expression was induced during the last 24 hours of siRNA treatment. Then, cells were harvested and analyzed for myc-Plk4-WT expression by immunoblotting against the indicated proteins. **(B)** Myc-Plk4-WT was expressed in HEK 293T cells together with FLAG vector or FLAG-βTrCP. Cells were harvested and protein levels analyzed by immunoblotting. Data kindly provided by J. Westendorf.

4.3.3 Plk4 Autophosphorylation Controls Its Degradation

βTrCP functions as the F-box adaptor protein within the SCF (Skp1-Cul1-F-box) E3 ubiquitin ligase to recognize and recruit ubiquitination substrates through direct interaction. In line with this and to extend the above observation that βTrCP regulates Plk4 protein levels, we next investigated whether βTrCP interacts with Plk4 to catalyze its degradation, and if so how this interaction is controlled.

4.3.3.1 Plk4 and βTrCP Interact Directly

To reveal whether βTrCP interacts directly with Plk4, co-immunoprecipitation experiments of overexpressed Plk4 and βTrCP from HEK 293T cells were performed. This revealed that wild-type Plk4 readily interacted with βTrCP, regardless of which protein was used as bait (Figure 18). Identical results were obtained in *in vitro* binding assays utilizing wild-type Plk4 isolated from cells and *in vitro* translated βTrCP (data not shown).

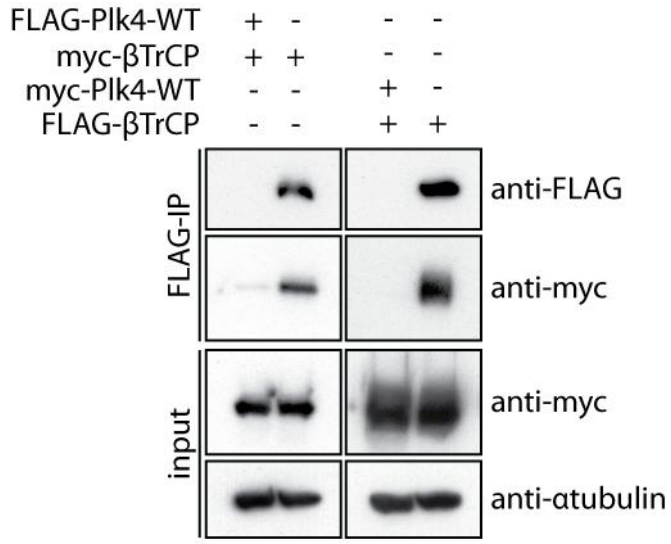

Figure 18. Plk4 and βTrCP interact biochemically. HEK 293T cells were co-transfected for 24 hours with Plk4 and βTrCP as indicated and anti-FLAG immunoprecipitations were performed. The co-immunoprecipitated proteins were detected by immunoblotting.

4.3.3.2 The Interaction of Plk4 and βTrCP Requires an Intact DSG Motif

βTrCP canonically recognizes a conserved DSGxx[S/T] motif (DSG motif) in its substrates to recruit them to the SCF complex for ubiquitination. This usually requires double phosphorylation of the DSG motif at the two phosphoacceptor residues (S/T), hence coining the term phosphodegron for the DSG motif. We therefore next explored whether the Plk4-βTrCP interaction is mediated through the, possibly phosphorylated, conserved DSGHAT motif in Plk4 (AA284-289). To this end, the phosphoacceptor residues within this motif, Ser285 and Thr289, were mutated to alanine or aspartatic acid to render an unphosphorylatable (Plk4-WT-DSG^{AA}) or a phosphomimetic DSG motif (Plk4-WT-DSG^{DD}), respectively. Interestingly, neither Plk4-WT-DSG^{AA} nor Plk4-WT-DSG^{DD} interacted with βTrCP in co-immunoprecipitation experiments (Figure 19). While this was expected for Plk4-WT-DSG^{AA}, the fact that Plk4-WT-DSG^{DD} also failed to bind βTrCP suggests that both mutations alter the biophysical properties of the DSG motif to disrupt the Plk4-βTrCP interaction, i.e. the lack of phosphorylation, and that the negative charge of aspartic acid as substitution of the phosphoacceptor residues does not suffice to mimic phosphorylation of the motif. Interestingly, both Plk4 mutants retained retarded electrophoretic mobility, arguing that Plk4 is phosphorylated at sites other than the DSG motif. In summary, we conclude that βTrCP binds Plk4 via the conserved DSG motif in Plk4.

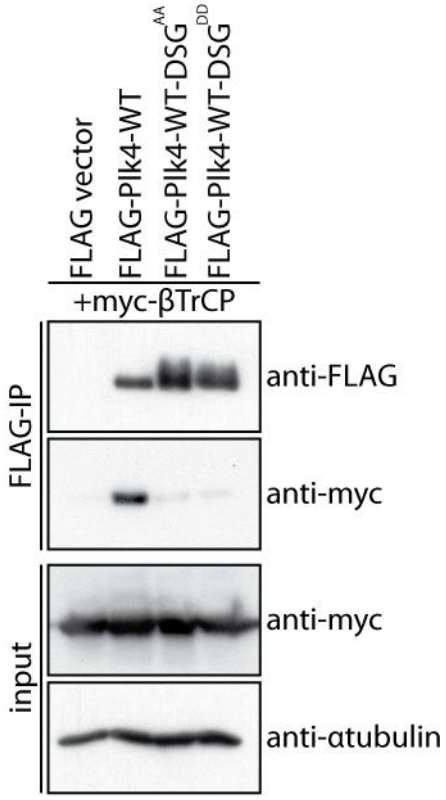

Figure 19. The interaction between Plk4 and βTrCP requires an intact DSG motif. Myc-βTrCP was co-expressed with FLAG-Plk4-WT, FLAG-Plk4-WT-DSG^{AA} or FLAG-Plk4-WT-DSG^{DD} in HEK 293T cells. Cell extracts were subjected to anti-FLAG immunoprecipitations and immunoprecipitates were probed for the indicated proteins by immunoblotting.

4.3.3.3 Plk4 Autophosphorylation is Required for its Interaction with βTrCP

After having established that βTrCP and Plk4 interact via the DSG motif and that the phosphoacceptor residues within this motif are crucial for binding, we next set out to assess whether the interaction of Plk4 and βTrCP indeed depends on phosphorylation, as is known for the other SCF$^{\beta TrCP}$ substrates (reviewed in Frescas and Pagano 2008). To this end, the Plk4-βTrCP complex was co-immunoprecipated from HEK 293T cells and treated with either buffer or λ-phosphatase (λPPase). Dephosphorylation of the complex relieved the retarded electrophoretic mobility of myc-Plk4-WT (Figure 20A), confirming that Plk4 is a phosphoprotein *in vivo* as suggested previously (Yamashita *et al.*, 2001). Most importantly, λPPase treatment disrupted the interaction of Plk4 and βTrCP (Figure 20A), indicating that phosphorylation is indeed required for the association of Plk4 and βTrCP.

The previous experiment had revealed that phosphorylation of the DSG motif seems to be a prerequisite for βTrCP binding and that dephosphorylation of Plk4 disrupts

its interaction with βTrCP. We therefore asked whether kinase-dead Plk4 which lacks phosphorylation sites intrinsic to Plk4 kinase activity but retains Plk4-independent phosphorylation sites binds to βTrCP. Indeed, loss of Plk4 kinase activity extensively reduces βTrCP binding, but still retains faint binding capacity when compared to the DSG-mutant (Figure 20B). This clearly demonstrates that Plk4 autophosphorylation activity is required for βTrCP binding.

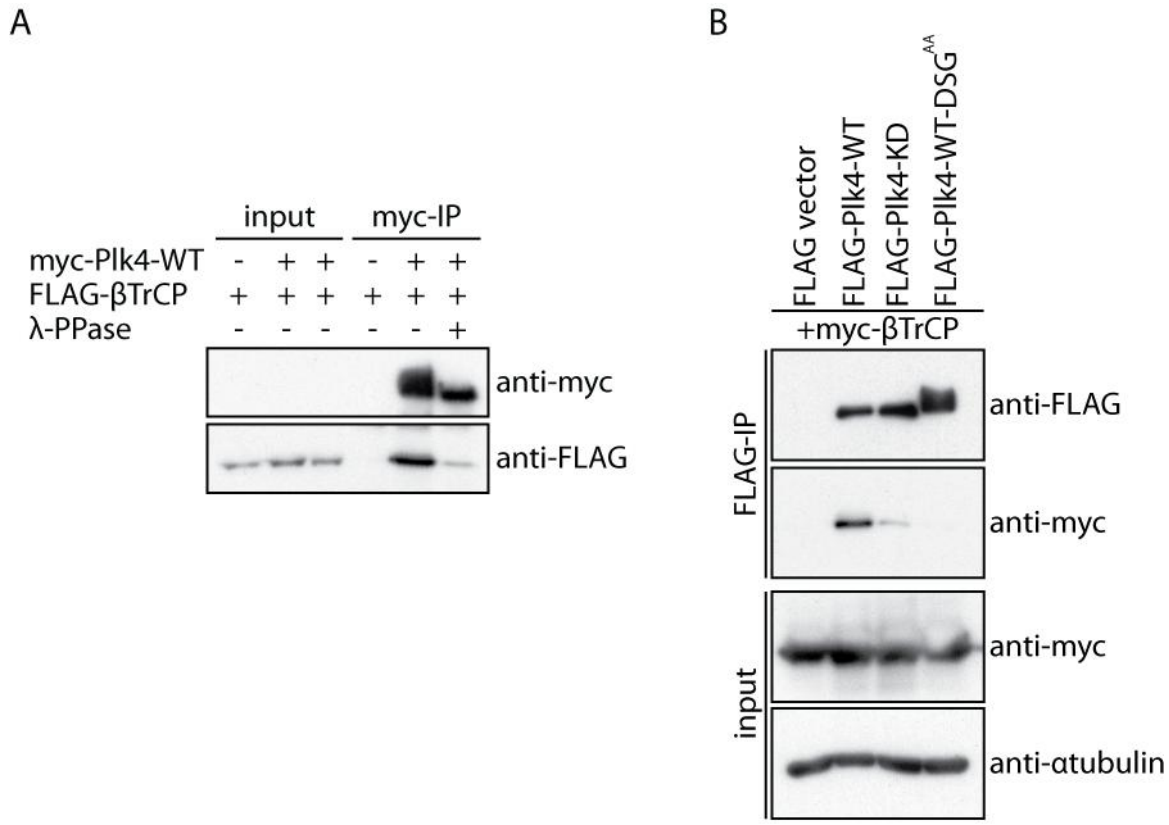

Figure 20. The interaction of Plk4 and βTrCP requires Plk4 autophosphorylation. (A) FLAG-βTrCP and myc-Plk4-WT were co-expressed in HEK 293T cells. Anti-myc immunoprecipitations were performed and immunoprecipitates treated with λ-phosphatase (λPPase) where indicated. The co-immunoprecipitated proteins were detected by immunoblotting. **(B)** Myc-βTrCP and FLAG-Plk4-WT, FLAG-Plk4-KD or FLAG-Plk4-WT-DSG^{AA} were co-expressed in HEK 293T cells. Anti-FLAG immunoprecipitations were performed and immunoprecipitates were probed for the indicated proteins by immunoblotting.

4.3.3.4 Plk4 Autophosphorylation is Required for its Ubiquitination and Degradation

As the adaptor molecule of the SCF complex, βTrCP recruits proteasome substrates for ubiquitination. It is therefore plausible that βTrCP mediates degradation of Plk4 by facilitating its ubiquitination and subsequent degradation by the 26S proteasome. Accordingly, perturbed interaction of Plk4 and βTrCP should result in reduced ubiquitination and degradation of Plk4. Supporting this idea, the Plk4 mutants which have

been described to be unable to bind to βTrCP, kinase-dead Plk4 (due to its lack of autophosphorylation activity) and Plk4-WT-DSG^{AA} (due to disruption of the DSG motif), were ubiquitinated to a lesser extent than wild-type Plk4 *in vivo* (Figure 21A). Identical results were obtained in an *in vitro* ubiquitination assay arguing against the co-precipitation of other ubiquitinated proteins (Figure 21B).

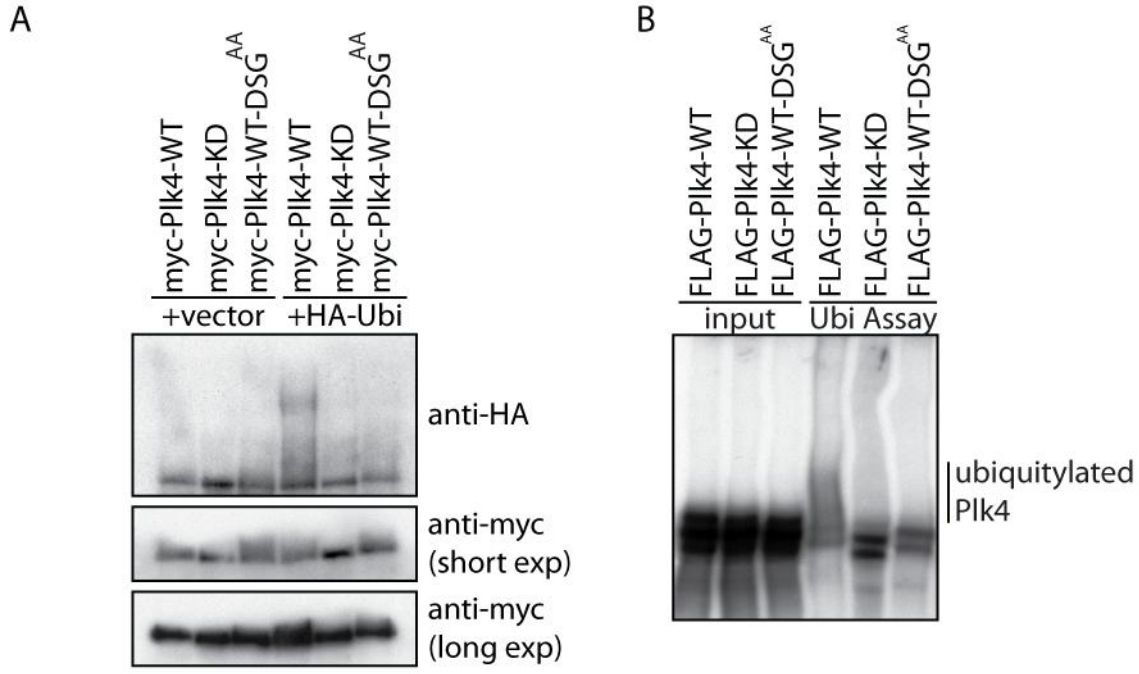

Figure 21. Plk4 autophosphorylation is required for efficient ubiquitination *in vivo* and *in vitro*. (A) Myc-Plk4-WT, myc-Plk4-KD or myc-Plk4-DSG^{AA} was co-expressed for 24 hours with HA vector or HA-ubiquitin. Cell extracts were subjected to anti-myc immunoprecipitations and probed by immunoblotting for the indicated proteins. **(B)** [^{35}S]-methionine labeled, *in vitro* translated FLAG-Plk4-WT, FLAG-Plk4-KD or FLAG-Plk4-WT-DSG^{AA} was subjected to *in vitro* ubiquitination assays. The presence of ubiquitinated Plk4 was assessed by autoradiography.

One would expect that lack of ubiquitination should stabilize Plk4 by protecting it from degradation via the 26S proteasome. Indeed, while Plk4-WT was degraded in cells treated with cycloheximide to inhibit protein synthesis for up to 8 hours, Plk4-KD was stabilized to a similar extent as Plk4-WT-DSG^{AA} (Figure 22). Intriguingly, no further decrease in Plk4-WT protein levels occurred between 4 and 8 hours of cycloheximide treatment, suggesting that a certain Plk4 fraction is resistant to degradation.

Together, these data suggest that Plk4 kinase activity is necessary for its interaction with βTrCP and, consequently, its polyubiquitination and subsequent degradation by the 26S proteasome.

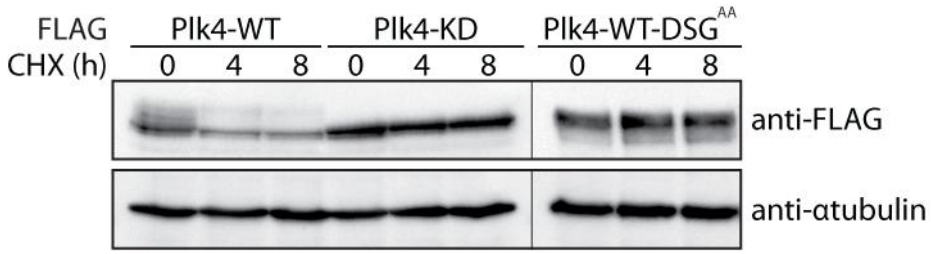

Figure 22. Kinase-dead Plk4 is stabilized comparable to a DSG-mutant of Plk4. FLAG-Plk4-WT, FLAG-Plk4-KD or FLAG-WT-DSG^{AA} was expressed in HEK 293T cells before protein synthesis was blocked by cycloheximide. Cells were harvested at the indicated time points and protein levels analyzed by immunoblotting. Data kindly provided by J. Westendorf.

4.3.4 Plk4 *trans*-Autophosphorylation Controls its Degradation and Centriole Number

The above finding that excess kinase-dead Plk4 triggers centriole overduplication in the presence of endogenous Plk4 fostered the idea that kinase-dead Plk4 may cause centriole overduplication through sequestration of βTrCP. Yet, the finding that Plk4-KD cannot interact with βTrCP argues against this possibility. This led us to explore an alternative model involving dimerization and *trans*-autophosphorylation of Plk4 to explain centriole overduplication in the presence of excess kinase-dead Plk4.

4.3.4.1 Plk4 Autophosphorylates Itself in *trans*

Plk4 has previously been shown to dimerize via its C-terminal coiled-coil region (Leung *et al.*, 2002; Habedanck *et al.*, 2005), yet whether this depends on Plk4 kinase activity was unknown. We therefore assessed whether Plk4 dimerization capacity is retained in the absence of Plk4 autophosphorylation (Figure 23). To this end, differentially tagged wild-type and kinase-dead Plk4 were co-overexpressed in HEK 293T cells and assayed for their ability to co-immunoprecipitate. Plk4 dimerization was observed regardless of its kinase activity, as kinase-dead Plk4 interacted with wild-type Plk4 as well as kinase-dead Plk4. Furthermore, both Plk4-KD and Plk4-KD-DSG^{AA} were phosphorylated by wild-type Plk4, manifested by the retarded electrophoretic mobility of kinase-dead Plk4 upon co-immunoprecipitation with wild-type Plk4 (Figure 23). This clearly demonstrates that Plk4 *trans*-autophosphorylates itself, also at sites distinct from the DSG motif (see also Sillibourne *et al.*, 2010).

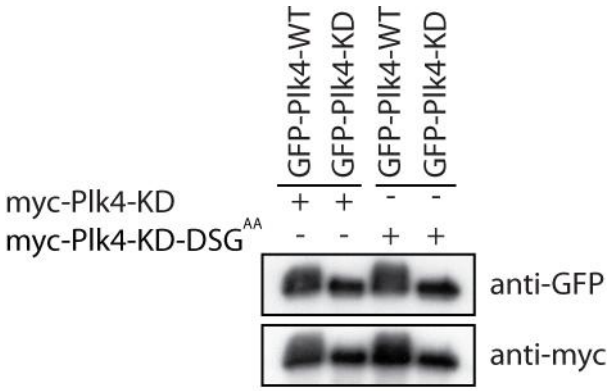

Figure 23. Plk4 autophosphorylates itself *in trans* and dimerizes regardless of kinase activity. Myc-Plk4-KD or myc-Plk4-KD-DSG$^{\ddot{A}A}$ was co-expressed for 24 hours with GFP-Plk4-WT or GFP-Plk4-KD and immunoprecipitated with anti-myc antibodies. The immunoprecipitates were subjected to an *in vitro* kinase assay which was analyzed by immunoblotting.

4.3.4.2 An N-terminal Truncation of Plk4 Causes Centriole Overduplication

The data above show that wild-type Plk4 is capable of *trans*-autophosphorylating kinase-dead Plk4. We therefore next asked whether *trans*-autophosphorylation plays a role in modulating the degradation of Plk4. To this end we searched for Plk4 fragments differing in their ability to autophosphorylate (Figure 25A), interact with βTrCP (Figure 25B) and dimerize (Figure 25C). Plk4$^{1\text{-}608}$ is active as a kinase and interacts with βTrCP but does not dimerize due to truncation of its C-terminus. Plk4$^{609\text{-}970}$, on the other hand, is kinase inactive and does interact with βTrCP due to truncation of its kinase domain. Yet, Plk4$^{609\text{-}970}$, which comprises the cryptic polo box, dimerizes with wild-type Plk4 via its coiled-coil domain (see also Leung *et al.,* 2002; Habedanck *et al.,* 2005).

The above-mentioned Plk4 fragments were then overexpressed in U2OS cells and assayed for their ability to trigger centriole overduplication. Remarkably, Plk4$^{609\text{-}970}$ caused strong centriole overduplication, occasionally resulting in the rosette-like arrangement of procentrioles, whereas Plk4$^{1\text{-}608}$ failed to do so (Figure 25). This was reminiscent of centriole overduplication triggered by overexpression of wild-type or kinase-dead Plk4 and accordingly fostered the hypothesis that excess kinase-dead Plk4 is able to cause centriole overduplication, provided that its ability to dimerize with endogenous Plk4 is preserved.

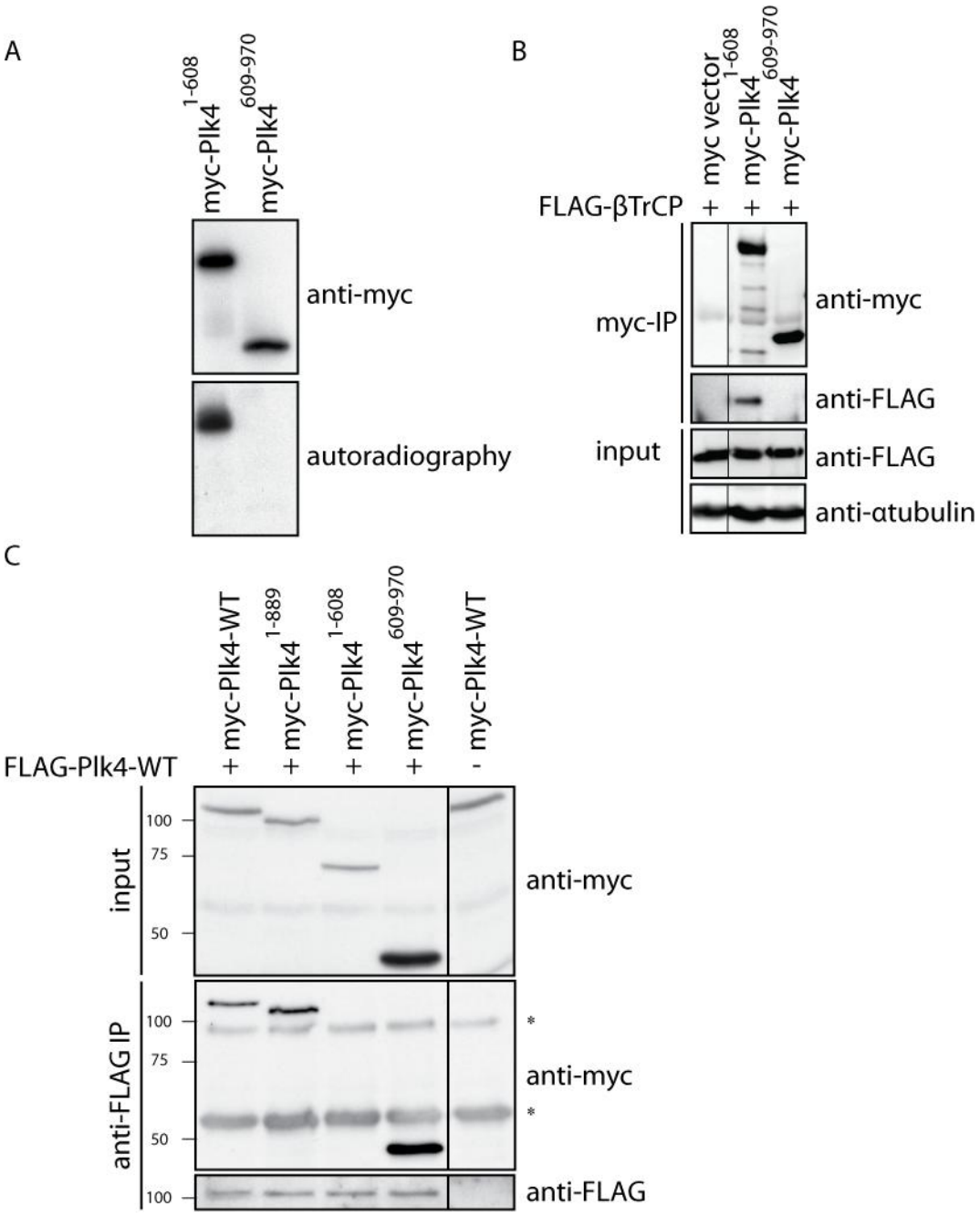

Figure 24. Myc-Plk4$^{1\text{-}608}$ and myc-Plk4$^{609\text{-}970}$ display differential properties regarding kinase activity, βTrCP binding and dimerization with Plk4-WT. (A) Myc-Plk4$^{1\text{-}608}$ or myc-Plk4$^{609\text{-}970}$ was overexpressed for 24 hours in HEK 293T cells. The overexpressed proteins were immunoprecipitated with anti-myc antibodies and subjected to *in vitro* kinase assays assay in the presence of γ-[^{32}P]-ATP. The kinase assay was analyzed by immunoblotting (upper panel) and autoradiography (lower panel). **(B)** Myc vector, myc-Plk4$^{1\text{-}608}$ or myc-Plk4$^{609\text{-}970}$ was co-overexpressed with FLAG-βTrCP for 24 hours in HEK 293T cells and anti-myc immunoprecipitations were performed. The assay was analyzed by immunoblotting against the indicated proteins. **(C)** Myc-Plk4-WT, myc-Plk4$^{1\text{-}889}$, myc-Plk4$^{1\text{-}608}$, myc-Plk4$^{609\text{-}970}$ or empty vector was co-overexpressed with FLAG-βTrCP for 24 hours in HEK 293T cells before anti-FLAG immunoprecipitations were performed. The immunoprecipitated proteins were analyzed by immunoblotting against the indicated proteins. Asterisks mark unspecific bands. Data presented in (C) was kindly provided by J. Westendorf.

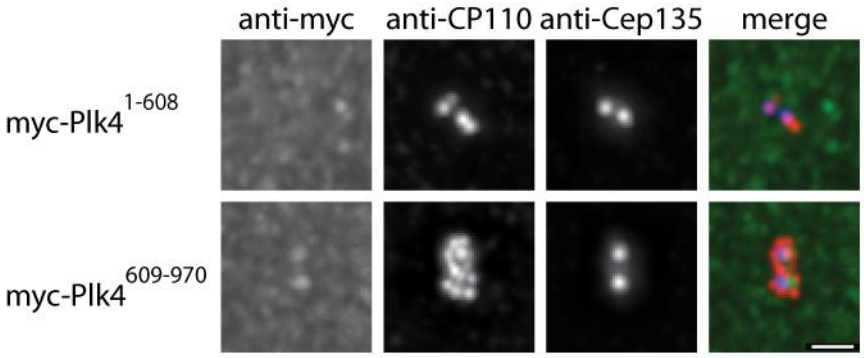

Figure 25. Myc-Plk4$^{609-970}$ causes centriole overduplication. U2OS cells were transfected with myc-Plk4$^{1-608}$ or myc-Plk4$^{609-970}$ for 48 hours. Cells were stained for the myc-epitope (green), CP110 (red) and Cep135 (blue). Scale bar: 1 µm.

4.3.4.3 Plk4 Autophosphorylation in *trans* Restores βTrCP Binding to Plk4-KD

The above data lead us to conclude that excess Plk4-KD triggers centriole overduplication by virtue of its ability to (hetero-)dimerize with endogenous, active Plk4. If so, 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 $SCF^{\beta TrCP}$ by acting as a decoy. A corollary of this model is that autophosphorylation in *trans* should convert Plk4-KD to a βTrCP-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 βTrCP (DSG-WT or DSG^{AA}). In these experiments, the myc-tagged constructs served as bait for βTrCP-binding, whereas the FLAG-tagged constructs, competent to dimerize but incompetent to bind βTrCP, provided kinase activity. The ability of the immunoprecipitated complexes to bind to βTrCP was then analyzed via an *in vitro* binding assay. Co-expression of FLAG-Plk4-KD-DSG^{AA} with myc-Plk4-KD failed to restore βTrCP binding, as expected, considering the absence of *trans*-autophosphorylation. In stark contrast, co-expression of FLAG-Plk4-WT-DSG^{AA} with myc-Plk4-KD fully restored the binding of myc-Plk4-KD to βTrCP (Figure 26). This demonstrates that autophosphorylation in *trans* is required to confer βTrCP-binding properties to Plk4.

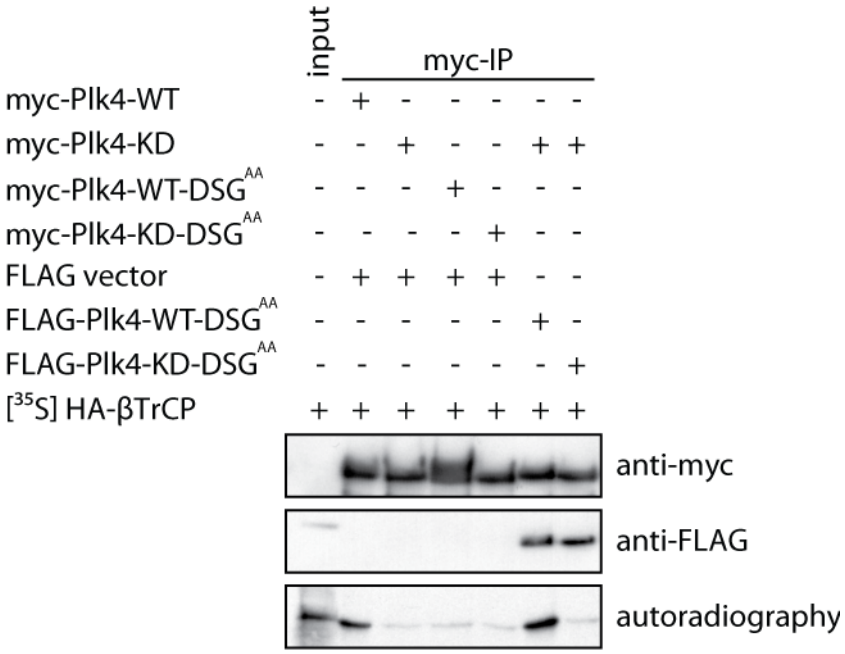

Figure 26. Plk4 autophosphorylation in *trans* restores βTrCP-binding capability to kinase-dead Plk4. HEK 293T cells were transfected with the indicated plasmids. Anti-myc immunoprecipitates were incubated with *in vitro* translated, [^{35}S]-methionine labeled βTrCP in an *in vitro* binding assay. The co-immunoprecipitated proteins were analyzed by immunoblotting and autoradiography.

4.3.4.4 Plk4 Autophosphorylation is Not Sufficient for βTrCP Binding

In agreement with the result that βTrCP binding requires Plk4 *trans*-autophosphorylation, two modes of regulation of Plk4-βTrCP interaction are conceivable (see also 5.4): either Plk4 *trans*-autophosphorylation is sufficient for βTrCP binding as it directly phosphorylates the DSG motif or Plk4 *trans*-autophosphorylation is required but not sufficient to promote βTrCP binding. In order to verify one of these two models, we next investigated whether Plk4 *trans*-autophosphorylation is also sufficient to trigger its βTrCP-mediated degradation. We thus devised an *in vitro* binding assay to investigate whether the autophosphorylation events conferred by Plk4 are sufficient to mediate βTrCP binding. Herein, bacterially expressed, purified MBP-tagged Plk4, which possessed autophosphorylation activity *in vitro* (Figure 27A), served as bait to precipitate *in vitro* translated, [^{35}S]-methionine labeled βTrCP. Yet, MBP-Plk4 did not bind βTrCP when incubated in binding buffer *in vitro* (Figure 27B), arguing that Plk4 autophosphorylation is not sufficient for βTrCP binding. This is enforced by the fact that the same binding assay performed after pre-incubation of MBP-Plk4 with cell extract allowed for βTrCP binding of kinase active Plk4 (Figure 27B). This indicated that additional factors and/or

phosphorylation events catalyzed by other protein kinases are responsible for catalyzing βTrCP binding.

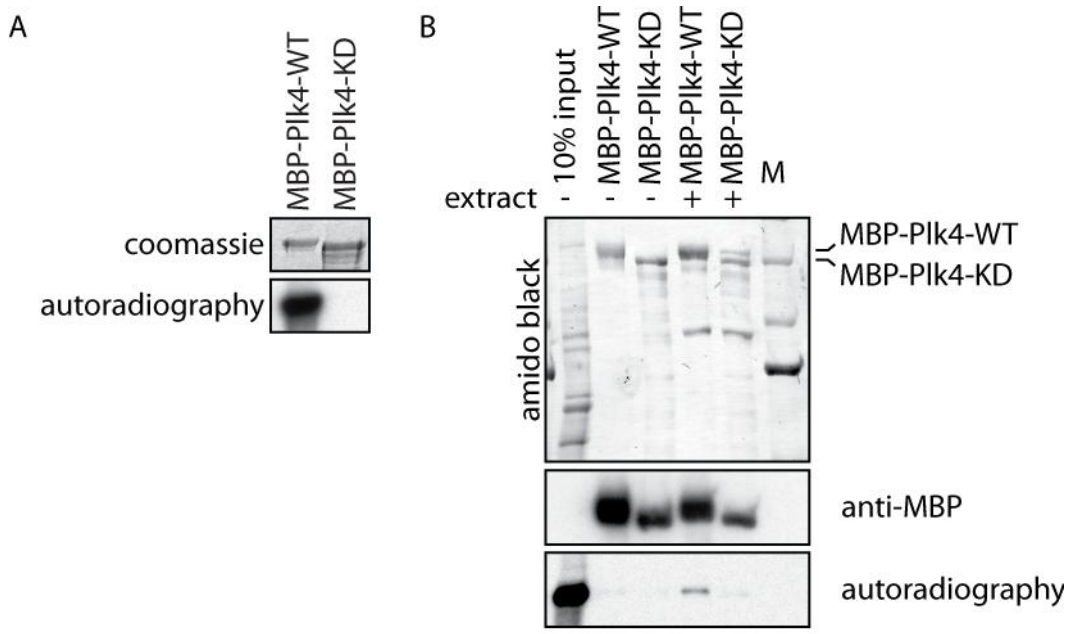

Figure 27. Recombinant wild-type Plk4 binds βTrCP after incubation with cell extract. (A) Purified, recombinant full-length wild-type and kinase-dead Plk4 were incubated in an *in vitro* kinase assay in the presence of γ-[^{32}P]-ATP. The kinase assay was analyzed by Coomassie staining (upper panel) and autoradiography (lower panel). **(B)** MBP-Plk4-WT or MBP-Plk4-KD were incubated with *in vitro* translated, [^{35}S]-methionine labeled βTrCP in an *in vitro* binding assay after incubation with or without extract from asynchronous HEK 293T cells. The precipitated proteins were analyzed by immunoblotting and autoradiography.

To confirm and extend the above observation that phosphorylation events other than Plk4 autophosphorylation are necessary for the Plk4-βTrCP interaction, we performed the binding assay as described above, but with Plk4 immunoprecipitated from HEK 293T cells as bait for βTrCP. As expected, wild-type Plk4 efficiently bound βTrCP while kinase-dead Plk4 and Plk4 dephosphorylated by λ-phosphatase (λPPase) treatment failed to do so (Figure 28). However, Plk4 that was rephosphorylated in an *in vitro* kinase assay after it had been dephosphorylated by λPPase treatment did not regain βTrCP binding. Together, this enforces the idea that Plk4 autophosphorylation is required, but not sufficient for βTrCP binding.

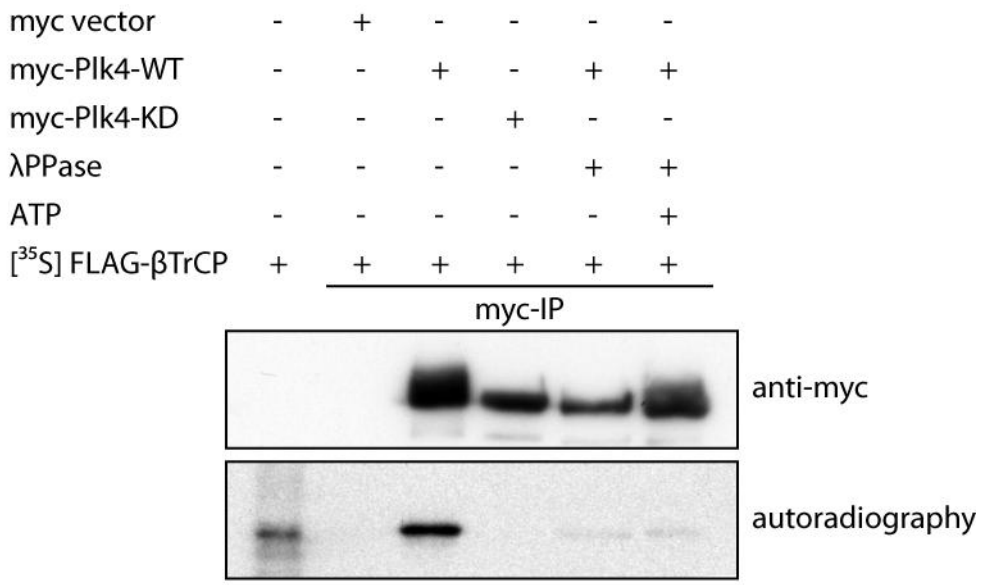

Figure 28. Rephosphorylated Plk4 does not bind βTrCP. Myc vector, myc-Plk4-WT or myc-Plk4-KD was overexpressed for 24 hours in HEK 293T cells and immunoprecipitated with anti-myc antibodies. The indicated immunoprecipitates were dephosphorylated with λ-phosphatase (λPPase) and rephosphorylated in an *in vitro* kinase assay before they were incubated with [^{35}S]-methionine labeled, *in vitro* translated FLAG-βTrCP in an *in vitro* binding assay. λPPase was inactivated by extensive washing of beads and phosphatase inhibitors. The assay was analyzed by immunoblotting (upper panel) and autoradiography (lower panel).

4.3.5 Does p38 Control the Interaction of Plk4 and βTrCP *in vitro*?

The above data revealed that Plk4 autophosphorylation is required, but not sufficient for βTrCP binding. This sparked the idea that Plk4 *trans*-autophosphorylation serves to create a docking site for a different, second kinase which in turn phosphorylates Plk4 on the phosphodegron in order to permit βTrCP binding. As a matter of fact, the 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 the recruitment of phosphodegron-directed kinases through phosphorylation-dependent docking sites.

4.3.5.1 Inhibition of p38 Disrupts the Interaction of Plk4 and βTrCP

To search for kinases that regulate βTrCP binding we screened a panel of protein kinase inhibitors for their ability to disrupt the Plk4-βTrCP interaction. To this end, overexpressed Plk4 was immunoprecipitated from HEK 293T cells which had previously been treated with different protein kinase inhibitors or DMSO as control for 2 hours before it was incubated with [^{35}S]-methionine labeled βTrCP in an *in vitro* binding assay.

Interestingly, of the nine kinase inhibitors used only SB202190, an inhibitor of MAP kinase p38, significantly reduced βTrCP binding to Plk4 (Figure 29)

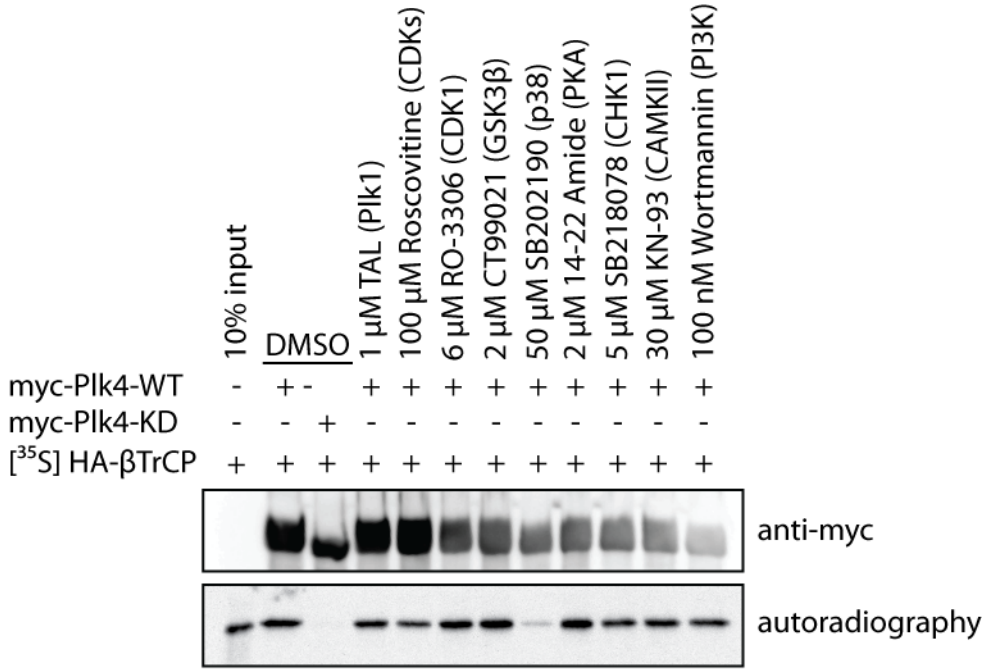

Figure 29. SB202190 inhibits binding of Plk4 and βTrCP. HEK 293T cells were transfected for 24 hours with myc-Plk4-WT or myc-Plk4-KD and treated for 2 hours with the indicated protein kinase inhibitors or DMSO as control before immunoprecipitations with myc-antibodies were performed. The immunoprecipitates were incubated with [^{35}S]-methionine labeled, *in vitro* translated HA-βTrCP in an *in vitro* binding assay. The bound proteins were analyzed by immunoblotting (upper panel) and autoradiography (lower panel).

Utilizing different concentrations of the p38 inhibitor, SB202190, as described above, we observed maximum inhibition at 20 µM (Figure 30, left panel). A second, independent p38 inhibitor, SB203580, showed a similar disruption of the Plk4-βTrCP interaction at 10 µM (Figure 30, right panel). Consequently, the disruption of the Plk4-βTrCP interaction by two independent p38 kinase inhibitors indicates that p38 kinase activity seems to be required for interaction of Plk4 and βTrCP.

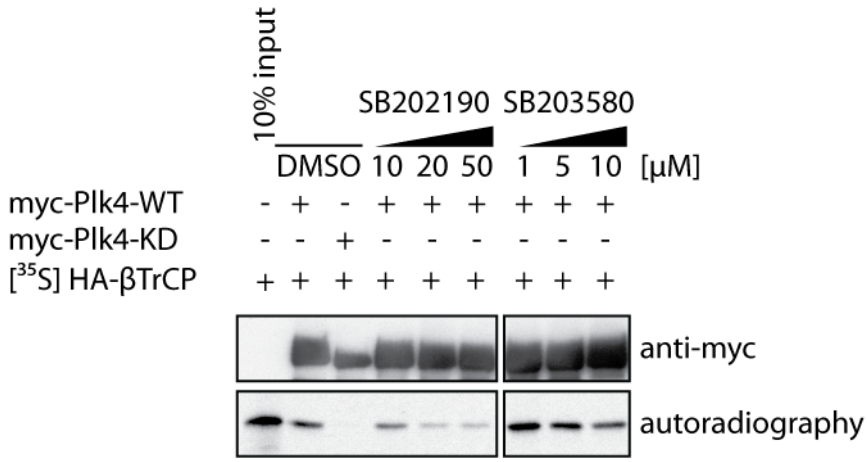

Figure 30. Small molecule inhibition of p38 disrupts interaction of Plk4 and βTrCP. HEK 293T cells were transfected with wild-type or kinase-dead Plk4 for 24 h before being treated with increasing concentrations of two different p38 inhibitors (SB202190, right panel; SB203580, left panel) or DMSO for 2 hours. Anti-myc immunoprecipitations were performed and the immunoprecipitated Plk4 was incubated with *in vitro* translated, [^{35}S]-methionine labeled βTrCP in an *in vitro* binding assay. Analysis of bound proteins was carried out by immunoblotting (upper panel) and autoradiography (lower panel).

4.3.5.2 p38 Inhibitors Do Not Perturb Plk4 Autophosphorylation

It was conceivable that the p38 inhibitors abrogated Plk4 kinase activity and thereby affected βTrCP binding. To exclude this possibility we performed an *in vitro* kinase assay in the presence of SB202190 and SB203580 to analyze its effect on Plk4 autophosphorylation. This revealed that neither SB202190 nor SB203580 inhibited autophosphorylation activity of overexpressed Plk4 immunoprecipitated from HEK 293T cells (Figure 31A) or recombinant Plk4 purified from *E. coli* (Figure 31B). The disruption of βTrCP binding to Plk4 by either inhibitor is thus not an effect of reduced Plk4 kinase activity.

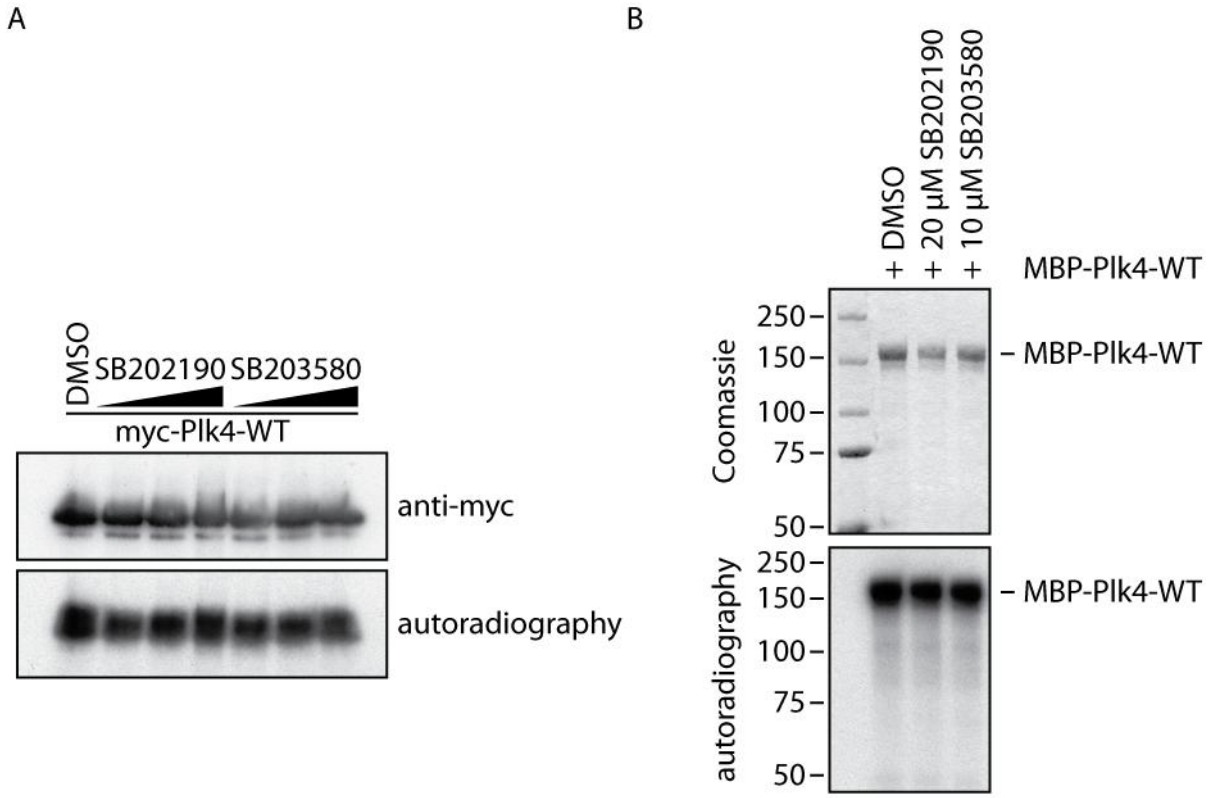

Figure 31. SB202190 and SB203580 do not inhibit Plk4 autophosphorylation. (A) HEK 293T cells were transfected for 24 hours with myc-Plk4-WT and treated with SB202190 (1 µM, 5 µM, 20 µM), SB203580 (1 µM, 5 µM, 20 µM) or DMSO as control. The overexpressed proteins were immunoprecipitated with anti-myc antibodies and subjected to *in vitro* kinase assays in the presence of γ-[^{32}P] ATP and the respective p38 inhibitor. **(B)** Recombinant MBP-Plk4-WT purified from *E. coli* was subjected to an *in vitro* kinase assay in the presence of DMSO, 20 µM SB202190 or 10 µM SB203580 and γ-[^{32}P] ATP.

4.3.5.3 Absence of p38 Activity Does Not Lead to Centriole Overduplication *in vivo*

The above experiments revealed that two independent p38 inhibitors disrupted the Plk4-βTrCP interaction without influencing Plk4 autophosphorylation activity. This prompted the question whether p38 kinase activity is also required for the interaction of Plk4 and βTrCP *in vivo*. In line with the observation that loss of βTrCP leads to increased Plk4 protein levels and centriole overduplication, reduced p38 activity should have the same phenotypic manifestation if it regulates Plk4-βTrCP binding. We therefore analyzed centriole numbers after inhibition of p38 for 48 hours with the described small molecule inhibitors SB202190 and SB203580 in asynchronous U2OS cells. Cells treated with 20 µM SB202190 or 10 µM SB203580 showed no difference to DMSO-treated control cells; neither in Plk4 localization to the centrioles nor centriole numbers. At the same time nuclear morphology and cell cycle progression, as measured by DAPI staining, was also

normal (data not shown). This clearly argues against a role of p38 in regulating Plk4 protein levels *in vivo*.

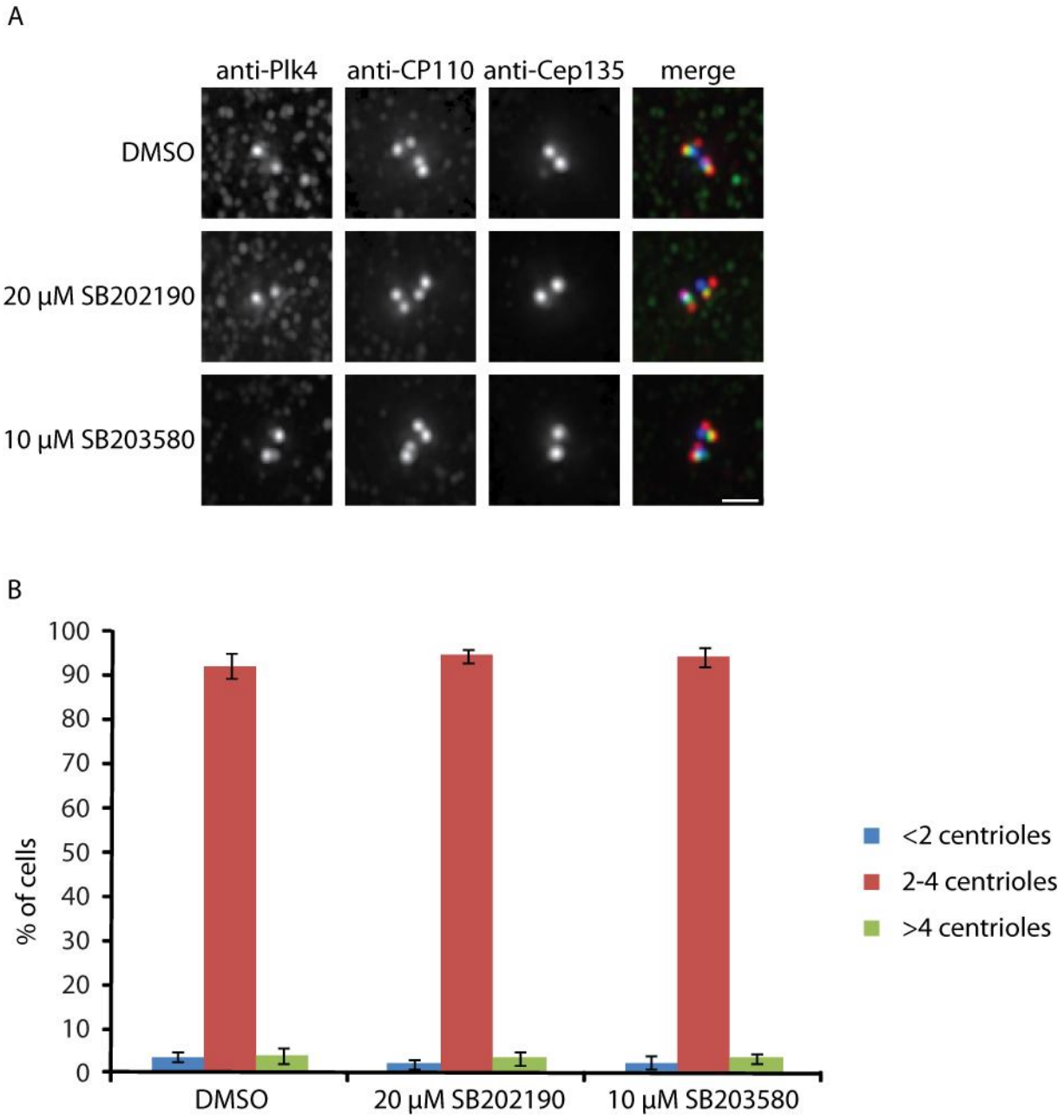

Figure 32. Small molecule inhibition of p38 does not perturb centriole duplication. (A) U2OS cells were treated for 48h with DMSO, 20 µM SB202190 or 10 µM SB203580. Cells were stained for Plk4 (green), CP110 (red) and Cep135 (blue). Scale bar: 1 µm. **(B)** Percentage of cells treated as described in (A) and grouped by the number of centrioles counted via CP110 staining. Data of three independent experiments (n = 100) are shown. Error bars denote s.e.m.

p38 exists in four isoforms (α, β, γ, δ) of which p38α and p38β have been described to be present in HEK 293T and HeLa cells (Jiang and Struhl 1998). Both of these are inhibited by the p38 inhibitors SB202190 and SB203580 (Karaman *et al.*, 2008)

and p38α has been described to be at the centrosome in its active phosphorylated form (Cha *et al.,* 2007; Lee *et al.,* 2010). We hence chose to deplete p38α in asynchronously growing U2OS cells for 72 hours by transfection of siRNA oligonucleotides in order to corroborate the above finding that small molecule inhibition of p38 does not influence centriole numbers. As positive control for disturbed Plk4 degradation and subsequent centriole overduplication we utilized βTrCP depletion. As reported above, βTrCP depletion leads to a significant increase in Plk4 protein levels at the centrioles and the number of cells with more than 4 centrioles compared to control-depleted cells (siGL2; Figure 33A,C). p38α depletion which was monitored by immunoblotting (Figure 33B), on the other hand, did not alter centriolar Plk4 protein levels or centriole numbers (Figure 33A,C).

In summary, neither small molecule inhibition of p38α and p38β nor siRNA-mediated depletion of p38α yielded any visible effect on Plk4 protein levels or centriole number in asynchronously growing U2OS cells. Consequently, the effect of small molecule p38 inhibition on the Plk4-βTrCP interaction observed *in vitro* could not be reproduced *in vivo*. We hence conclude that p38 most likely does not regulate Plk4 protein levels in dividing cells.

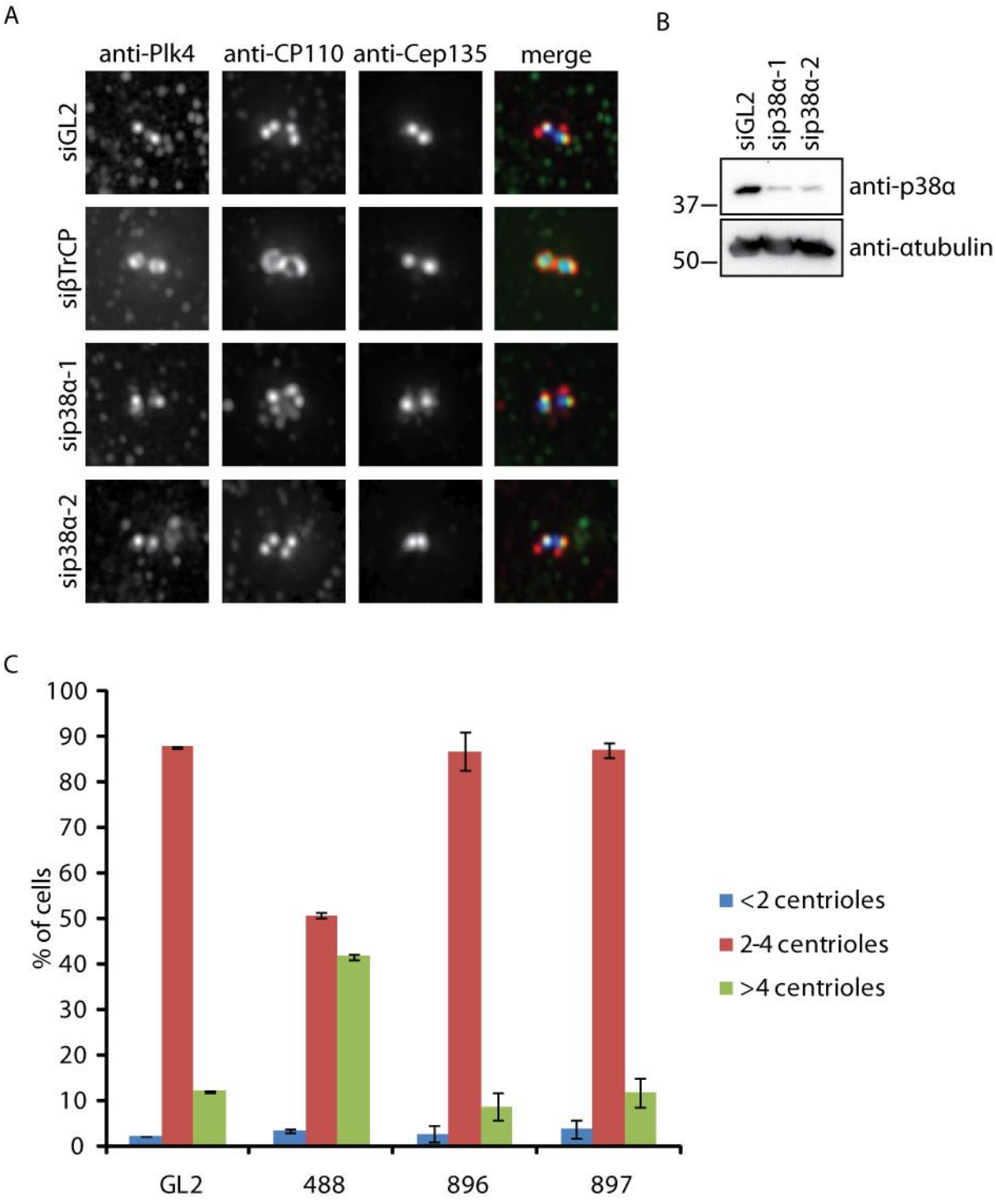

Figure 33. siRNA-mediated depletion of p38α does not perturb centriole duplication. (A) U2OS cells were transfected for 72 hours with siRNA oligonucleotides targeting GL2, βTrCP or two independent oligonucleotides targeting p38α before cells were stained against Plk4 (green), CP110 (red) and Cep135 (blue). Scale bar: 1 µm. **(B)** U2OS cells were transfected with siRNA oligonucleotides as described in (A) but processed for immunoblotting. **(C)** Percentage of cells treated as described in (A) and grouped by the number of centrioles counted via CP110 staining. Data of three independent experiments (n = 100) are shown. Error bars denote s.e.m.

5 DISCUSSION

The regulation of the centrosome duplication cycle has gained increasing scientific attention in recent years due to accumulating evidence that aberrations in centrosome numbers are causally linked to cancer development (reviewed in Nigg 2002; Godinho *et al.,* 2009). The concerted efforts to unravel the molecular architecture of the regulatory mechanisms controlling centriole duplication have led to the discovery of many key proteins involved in this process. The breakthrough came with the discovery that the kinase activity of Plk4 is pivotal to copy number control (Habedanck *et al.,* 2005). Since then tremendous efforts have been undertaken to understand how Plk4 fulfills this function. Nevertheless, the fundamental mechanisms of how precise regulation of Plk4 kinase activity is achieved to ensure faithful centriole duplication has not been unraveled.

Here, we have gained insight into Plk4's role in controlling centriole duplication. We demonstrate that Plk4 is subject to βTrCP-dependent proteasomal degradation. Active Plk4 promotes its own degradation by catalyzing βTrCP binding through *trans*-autophosphorylation within homodimers. While *trans*-autophosphorylation is required, it is not sufficient for this process. Unexpectedly, we found that excess kinase-dead Plk4 leads to centriole overduplication, provided that endogenous wild-type Plk4 is present. Our data indicate that this phenotype results from disruption of Plk4 *trans*-autophosporylation by kinase-dead Plk4, which then shields endogenous Plk4 from recognition by βTrCP.

5.1 Plk4 Kinase Activity is Essential for Centriole Duplication

The initial description of Plk4 as the key regulator of copy number control revealed that Plk4 kinase activity is essential for centriole duplication (Habedanck *et al.,* 2005). Puzzlingly, the introduction of excess kinase-dead Plk4 also lead to significant centriole overduplication, similar to excess active Plk4, and these findings have subsequently been confirmed by others (Holland *et al.,* 2010). In line with the idea that supernumerary centriole numbers might arise via cell division failures and due to the fact that kinase-dead induced centriole overduplication was suppressed in S phase-arrested cells, it was suggested that kinase-dead Plk4 might cause occasional cell division failures which result in the doubling of centriole numbers. This was further corroborated by the finding that

reduced Plk4 protein levels in heterozygous $Plk4^{+/-}$ mice also exhibited centrosome amplification (Ko *et al.*, 2005). However, centriole duplication is also efficiently triggered by stable overexpression of kinase-dead Plk4 even in S phase-arrested cells. Furthermore, centriole duplication induced by excess kinase-dead Plk4 is indistinguishable from wild-type Plk4-induced centriole overduplication. In both cases procentrioles appear in a rosette-like arrangement around the pre-existing centriole which has been demonstrated to be the result of a violation of centriole copy number control due to increased Plk4 kinase activity (Kleylein-Sohn *et al.*, 2007). This clearly refutes the notion that kinase-dead Plk4-induced centriole overduplication stems from cell division failures. Moreover, the analysis of centriole numbers with the novel centriolar marker CP110 revealed that transient overexpression of kinase-dead Plk4 also leads to centriole overduplication which is identical to canonical Plk4-induced centriole overduplication. CP110 decorates the distal end of centrioles, thereby allowing the detection of growing procentrioles at very early stages of centriole duplication. In absence of such a marker, previous studies were most likely unable to detect centriole duplication at such an early stage of centriole duplication.

Interestingly, kinase-dead Plk4 is not sufficient to drive centriole duplication as it relies on the presence of endogenous Plk4. This reaffirms that centriole duplication, as well as centriole overduplication, strictly requires kinase active Plk4 to catalyze the recruitment of the centriole duplication proteins for procentriole formation. Yet, it does not rationalize how kinase-dead Plk4 triggers centriole duplication in the presence of endogenous Plk4. One possible explanation is that excess kinase-dead Plk4 recruits a surplus of centriole duplication proteins independently of its kinase activity which would subsequently be phosphorylated by active Plk4 to trigger formation of supernumerary procentrioles. The enlargement of the PCM similarly triggers centriole overduplication even though this occurs via the *de novo* pathway (Loncarek *et al.*, 2008). Alternatively, conforming to the Slimb-dependent degradation of Plk4 in *Drosophila*, kinase-dead Plk4 could protect endogenous Plk4 from degradation and lead to an increase in the protein levels of endogenous Plk4. Mechanistically speaking, kinase-dead Plk4 could scavenge protein(s) required for the degradation of Plk4, for instance βTrCP, and endogenous Plk4 would then be stabilized beyond the threshold of centriole overduplication. Yet, this is refuted by the fact that kinase-dead Plk4 is unable to bind βTrCP (please refer to 4.3.3.3 and see also 5.3). The evidence gathered here points to a third possibility involving *trans*-autophosphorylation within heterodimers of endogenous and kinase-dead Plk4, also in the

context of βTrCP-mediated degradation of Plk4 which will be discussed later (please refer to section 5.3).

5.2 βTrCP Controls Centriole Numbers through Degradation of Plk4

The correlation of Plk4 protein levels with centriole numbers has fostered the concept that Plk4 activity is tightly regulated at the centrosome to ensure centriole copy number control. The small window of Plk4 activity within which faithful centriole duplication occurs may be achieved via a variety of mechanisms. A general scheme in the regulation of protein kinases is the interplay of phosphorylation and dephosphorylation by upstream kinases and phosphatases to control kinase activity (reviewed in Hunter 2007). In addition, the protein levels of the kinase may be directly regulated by its proteolysis via the ubiquitin-proteasome system.

Several lines of evidence implicate ubiquitination-mediated proteolysis in centriole copy number control. First and foremost, proteasome function is obligatory for faithful centriole duplication (Duensing *et al.,* 2007). Second, components of the E3 ubiquitin ligase complex SCF (Skp1-Cul1-F-box) have been shown to localize to the centrosome (Freed *et al.,* 1999) and to be required for constant centrosome numbers (Nakayama *et al.,* 2000; Wojcik *et al.,* 2000; Guardavaccaro *et al.,* 2003; Murphy 2003). In this line, the founding member of the F-box family, cyclin F, has recently been implicated in regulating centriole biogenesis as it was shown to catalyze degradation of CP110 (D'Angiolella *et al.,* 2010). Third, while this work was in progress, the protein levels of *Drosophila* Plk4 were shown to be directly regulated by the F-box protein Slimb (Cunha-Ferreira *et al.,* 2009; Rogers *et al.,* 2009). Yet, whether this mechanism is conserved from *Drosophila* to man was unclear, especially because fundamental differences in the regulation of *Drosophila* and human Plk4 have been demonstrated but not explained (Carvalho-Santos *et al.,* 2010).

In this study we could show that Plk4 protein levels are indeed regulated by βTrCP, the human homologue of Slimb. Correspondingly, inhibition of the proteasomal degradation of Plk4 either by general proteasome inhibition or βTrCP depletion leads to increased centrosomal Plk4 protein levels and supernumerary centrioles in the rosette-like arrangement of procentrioles around the pre-existing centriole, the phenotypic

manifestation of excess Plk4 (Habedanck *et al.,* 2005; Kleylein-Sohn *et al.,* 2007). A direct link between βTrCP and Plk4 protein levels could be affirmed by the fact that centriole overduplication upon βTrCP depletion depends on the presence of Plk4. Moreover, βTrCP and Plk4 interact directly via the conserved DSG motif of Plk4. Altogether this shows that the control of Plk4 protein levels by βTrCP is conserved from *Drosophila* to man. Additionally, the fact that ZYG-1, the functional analogue of Plk4 in *C. elegans*, also contains a DSG motif suggests that lin-23, the *C. elegans* homologue of βTrCP (Kipreos *et al.,* 2000), may regulate ZYG-1 protein levels and puts forth the intriguing idea that the mode of control over the protein levels of ZYG-1 and Plk4 is identical even though ZYG-1 and Plk4 most likely arose through convergent evolution (Carvalho-Santos *et al.,* 2010).

The importance of efficient βTrCP-mediated degradation is emphasized by the fact that βTrCP is deregulated in many cancers and βTrCP has hence been attributed oncogenic as well as tumor suppressor properties (reviewed in Frescas and Pagano 2008). A possible role for βTrCP in tumorigenesis is furthermore suggested by the fact that it is required for the timely degradation of many cell cycle regulators, e.g. Cdc25 (Busino *et al.,* 2003; Kanemori *et al.,* 2005) or Emi1 (Margottin-Goguet *et al.,* 2003; Peters 2003). Now, we and others (Cunha-Ferreira *et al.,* 2009; Rogers *et al.,* 2009; Holland *et al.,* 2010) contribute to this concept by demonstrating that deregulated βTrCP levels result in supernumerary centrosomes which may result in chromosomal instability (Ganem *et al.,* 2009), a hallmark of many tumors (Lengauer *et al.,* 1997; D'Assoro *et al.,* 2002; Nigg 2002; Sluder and Nordberg 2004).

5.3 Plk4 *trans*-Autophosphorylation Regulates its βTrCP-mediated Degradation

The revelation that human Plk4 is degraded by βTrCP uncovered how Plk4 protein levels are controlled to ensure faithful centriole duplication. Insight into how this process is controlled then came from the realization that Plk4 autophosphorylation is required for interaction with βTrCP and its subsequent ubiquitination and degradation. Similar results were also reported by others (Holland *et al.,* 2010) and are in good agreement with the idea that the activated conformation of a protein kinase is a prerequisite for initiating its

degradation (Kang *et al.,* 2000). However, Holland *et al.* reported that Plk4 with a non-phosphorylatable DSG motif (Plk4-WT-DSG$^{AA\ (S285A/T289A)}$) is stabilized to a lesser extent than kinase-dead Plk4. The authors rationalize this finding with their observations that a 24 amino acid region around the DSG motif is involved in βTrCP binding and that mutation of all 13 phosphoacceptor residues to alanine within this region stabilized Plk4 to a greater degree than the Plk4-WT-DSG^{AA} mutation (Holland *et al.,* 2010). Yet, the authors do not present a plausible rationalization for this effect. In this context, it is noteworthy that a Plk4 mutant carrying aspartic acids instead of the phosphorylatable residues in the DSG motif (Plk4-WT-DSG$^{DD\ (S285D/T289D)}$) does not interact with βTrCP. Possibly other phosphorylation events necessary for βTrCP recognition are prevented by this mutation. Alternatively, simple addition of negative charges to the DSG motif may not suffice to mimic the presence of phosphate-groups to allow βTrCP binding.

The requirement for Plk4 autophosphorylation in βTrCP binding refuted the previous working model that centriole overduplication upon overexpression of kinase-dead Plk4 may arise from direct sequestration of βTrCP by kinase-dead Plk4 and subsequent increase in Plk4 protein levels. More extensive investigation of the interplay between Plk4 autophosphorylation and its βTrCP-mediated degradation revealed that Plk4 kinase activity alone is not sufficient to cause centriole overduplication; it also has to retain centrosome localization and dimerization, confirming earlier results (Habedanck *et al.,* 2005). Apart from that, Plk4 fragments which localize to the centrosome and only contain the so-called cryptic polo box and are therefore kinase inactive, induce centriole overduplication with the rosette-like arrangement of procentrioles around the pre-existing centriole. Accordingly, the cryptic polo box of Plk4 should be involved in triggering centriole overduplication upon overexpression of kinase-dead Plk4. The cryptic polo box contains a coiled-coil domain which is required for both Plk4 dimerization and its centriolar localization (Leung *et al.,* 2002; Habedanck *et al.,* 2005). This fostered the idea that excess kinase-dead Plk4 is able to cause centriole overduplication, provided that its ability to dimerize with endogenous Plk4 is preserved. Further support for this came from the observation that the ability of kinase-dead Plk4 to bind βTrCP could be restored by *trans*-autophosphorylation, while lack of *trans*-autophosphorylation prevented this. A mechanism for activation-dependent protein degradation of a Ser/Thr protein kinase was first demonstrated for PKCη in which kinase activity acts as part of a regulatory feedback mechanism (Lu *et al.,* 1998). Comparable to Plk4, constitutively active PKCη can trigger

the degradation of a degradation-resistant, kinase-inactive PKCη polypeptide via *trans*-autophosphorylation.

The above demonstrated that autophosphorylation in *trans* is required to confer βTrCP-binding properties to Plk4 and led to a model explaining how excess kinase-dead Plk4 stabilizes endogenous Plk4 and thereby leads to centriole overduplication (Figure 34): Excess kinase-dead Plk4 (hetero-)dimerizes with endogenous Plk4 and thereby outcompetes endogenous Plk4. Kinase-dead Plk4 is consequently phosphorylated in *trans* by endogenous Plk4, but not *vice versa*: this catalyzes βTrCP-mediated ubiquitination and degradation of kinase-dead Plk4. At the same time the endogenous Plk4 polypeptide is left unscathed and ready to undergo another cycle of dimerization, *trans*-autophosphorylation and degradation of kinase-dead Plk4. This eventually increases endogenous Plk4 protein levels above the threshold of centriole overduplication and overrides centriole copy number control. In excellent agreement with this conclusion, wild-type Plk4 was shown to promote destruction of kinase-dead Plk4 through intermolecular phosphorylation (Holland *et al.*, 2010).

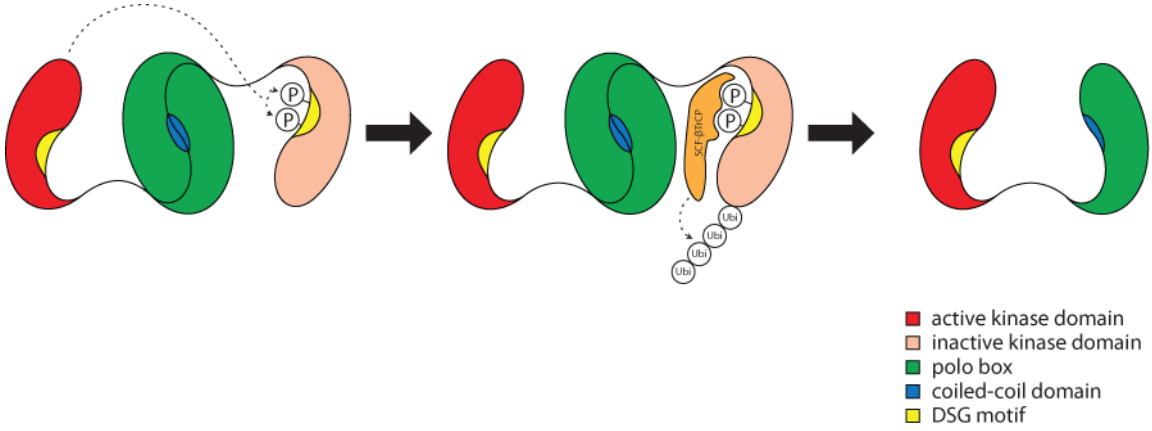

Figure 34. Model of how kinase-dead Plk4 stabilizes endogenous Plk4. Within heterodimers of active and kinase-dead Plk4, active Plk4 *trans*-autophosphorylates kinase-dead Plk4 and leads to its βTrCP-mediated degradation either through direct phosphorylation of the DSG motif or through recruitment of an additional kinase which then phosphorylates the DSG motif (for the sake of simplicity the latter possibility has been left out in this schematic). This leaves the active Plk4 molecule unscathed and free to dimerize; most likely with one of the excess kinase-dead Plk4 polypeptide. Hence, active Plk4 is protected from degradation and its levels will increase beyond the threshold of centriole overduplication.

5.4 Plk4 Kinase Activity is Not Sufficient for its βTrCP-mediated Degradation

According to the results discussed above, Plk4 seems to follow the general principles that apply to the recognition of activated protein kinases for ubiquitination and degradation by βTrCP (reviewed in Hunter 2007; Lu and Hunter 2009). *Trans*-autophosphorylation of a protein kinase may directly activate the DSG motif to create binding sites for E3 ligases. In some instances, both phosphates are added to the DSG motif by the same kinase, as has been shown for IκBα phosphorylation by IKK (Winston *et al.*, 1999). Alternatively, the generation of the phosphodegron requires the cooperative action of two kinases. One possibility is that the two phosphates within the DSG motif are added by two different kinases; one kinase acts as a priming kinase to recruit a second kinase, as is the case for β-catenin (Liu *et al.*, 2002). Alternatively, a priming phosphorylation creates a docking site which is distinct from the DSG motif to recruit a second kinase that phosphorylates the DSG motif. For instance CDK1 phosphorylation of Wee1 recruits Plk1 via its polo box which then creates the SCF phosphodegron (Watanabe *et al.*, 2004).

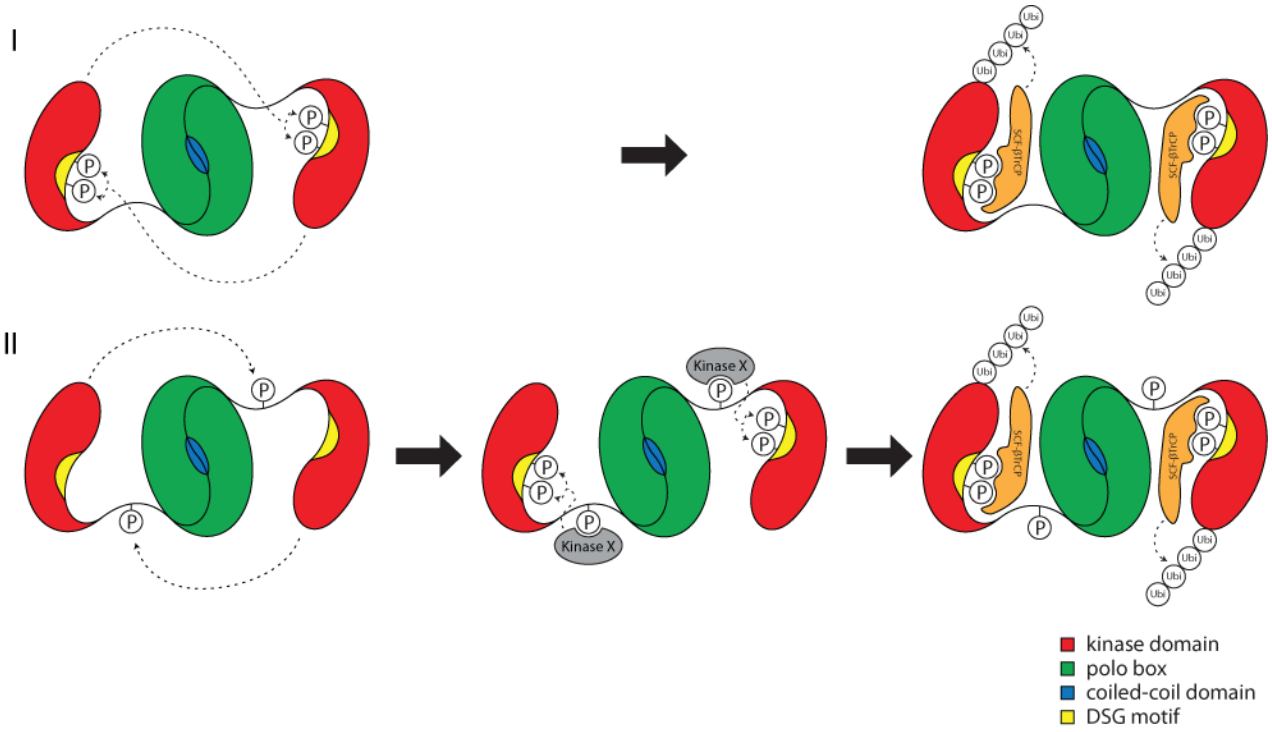

Figure 35. Two schematic models of how Plk4 *trans*-autophosphorylation may regulate βTrCP binding. According to model I, Plk4 autophosphorylation directly phosphorylates the DSG motif in *trans* and this is sufficient for βTrCP binding. Alternatively (model II), Plk4 autophosphorylation in *trans* creates a docking site for an unknown kinase X. In both cases, phosphorylation of the DSG motif is proposed to initiate the degradation of Plk4.

The requirement for Plk4 *trans*-autophosphorylation in βTrCP binding did not reveal whether *trans*-autophosphorylation is not just required, but also sufficient for Plk4-βTrCP binding. This raises the question which mode of action Plk4 follows. Is Plk4 *trans*-autophosphorylation sufficient to create the βTrCP phosphodegron or is a second kinase required for this process? *A priori*, it is possible that Plk4 *trans*-autophosphorylation directly activates the phosphodegron for βTrCP binding (Figure 35, model I). Alternatively, Plk4 might *trans*-autophosphorylate 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 (Figure 35, model II). In support of the latter possibility, the 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 the recruitment of phosphodegron-directed kinases through phosphorylation-dependent docking sites.

Further support for the involvement of an additional kinase in the regulation of the Plk4-βTrCP interaction stems from the fact that Plk4 *trans*-autophosphorylation did not confer βTrCP-binding capability to Plk4 *in vitro*; for wild-type Plk4 purified from eukaryotic or prokaryotic cells. In contrast, recombinant, wild-type Plk4 acquired βTrCP-binding capacity through incubation with cell extracts. Even though the latter result does not prove the involvement of an additional kinase, together with the other points of evidence, it strongly suggests that an additional kinase is required for βTrCP binding as proposed in the two-step model (Figure 35, model II). The mode of recruitment of the second kinase, however, remains unclear. *A priori*, concordant with the observations for other phosphodegrons, Plk4 autophosphorylation could directly create a docking site for another kinase. Alternatively, Plk4 autophosphorylation could also cause a conformational change which allows phosphorylation of the DSG motif by another kinase without the necessity of a docking site for the second kinase.

Initial insight into which kinase may cooperate with Plk4 *trans*-autophosphorylation to promote βTrCP binding resulted from a screen with various kinase inhibitors for their potency to inhibit the Plk4-βTrCP interaction. Surprisingly, out of all kinase inhibitors tested, only inhibition of the MAP kinase p38 reliably perturbed βTrCP binding. The two small molecules used (SB202190, SB203580) have been demonstrated to be potent and specific p38 inhibitors (Davies *et al.*, 2000; Bain *et al.*, 2007; Karaman *et*

al., 2008). Even though p38α has been described at the centrosome in its phosphorylated active state (Cha *et al.,* 2007; Lee *et al.,* 2010), small molecule inhibition of p38 or siRNA-mediated depletion of p38α did not have obvious effects on centriole numbers in dividing cells. This suggests that p38 may not be involved in regulating Plk4-βTrCP binding *in vivo*. Moreover, p38 is activated in response to stress conditions and proinflammatory cytokines (reviewed in Schaeffer and Weber 1999) and would therefore not be expected to be involved in the regulation of canonical cellular events. Additionally, it is expected that the kinase regulating Plk4 degradation should be cell cycle regulated in order to coordinate Plk4 protein levels with the centriole duplication cycle. We therefore emphasize that even though other kinase inhibitors targeting e.g. Plk1, GSK3-β or CDKs did not yield any effect, this does not preclude a role of these kinases in regulating Plk4 degradation, as experiments *in vivo* may yield different results. Especially Plk1 is a promising candidate for regulating Plk4 protein levels as many βTrCP substrates require Plk1 phosphorylation for efficient βTrCP binding and subsequent degradation, e.g. Wee1 (Watanabe *et al.,* 2004) and Erp1 (Liu and Maller 2005; Rauh *et al.,* 2005; Hansen *et al.,* 2006). Hence, future investigation will have to show whether *in vivo* experiments validate the results obtained after chemical inhibition of Plk1. In this context, it is important to note that inhibition of the above-mentioned kinases leads to cell cycle defects which would hinder the analysis of centriole overduplication. To circumvent this in the future, steps preceding the phenotypic manifestation of reduced Plk4 degradation, i.e. centriole overduplication, should be assessed.

In principle, mechanisms could also exist to counteract Plk4 degradation in order to locally and/or temporally increase Plk4 activity to trigger centriole duplication. *A priori*, Plk4, as any other βTrCP substrate, may be protected from degradation by spatially segregating it from βTrCP, e.g. sequestering of βTrCP or obstruction of the βTrCP binding site. Second, Plk4 may be actively protected from degradation by a phosphatase which removes phosphates critical for βTrCP recognition, e.g. at the DSG motif, or for docking of the second kinase X, implicated in regulating βTrCP binding. And third, the activity of the second kinase X towards Plk4 may be regulated in a spatio-temporal manner. The presence of a mechanism protecting a pool of Plk4 from degradation is justified by the fact that overexpressed Plk4 is only partly degraded in cycloheximide assays. Hence, it is important to devise methods to study endogenous Plk4 protein levels in order to understand whether endogenous Plk4 follows the same degradation kinetics as

overexpressed Plk4. This would also give further insight into the mechanisms regulating Plk4 degradation.

In conclusion, our data provides important mechanistic insight into the regulation of Plk4 protein levels. We provide a rational for the induction of centriole overduplication by excess kinase-dead Plk4 through *trans*-autophosphorylation by endogenous active Plk4. Furthermore, we suggest that Plk4 *trans*-autophosphorylation, albeit required, is not sufficient for its βTrCP-mediated degradation. This also raises interesting new questions. Future research should aim at exploring the timing of Plk4 degradation during the cell cycle and the identity of the kinase that is proposed here to contribute to control Plk4 stability.

6 MATERIALS AND METHODS

Plasmids and Cloning

Cloning of Plk4 and βTrCP1 cDNA has been described previously (Habedanck *et al.*, 2005; Chan *et al.*, 2008).

All cloning procedures were performed according to standard techniques as described in "Molecular Cloning: A Laboratory Manual" (Sambrook, 1989; 2nd edition) and "Current Protocols in Molecular Biology" (Wiley, 1999). Restriction enzymes were purchased from Fermentas (Burlington, Ontario, Canada) and ligation reactions were performed using T4 DNA ligase (NEB, Ipswich, MA). Plasmid purifications and DNA extractions from agarose gels were done as specified by the supplier (QIAGEN). Sequence mutations in Plk4 were inserted by using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions using specific primers. For a complete list of primers used in this study see Table 4. HA-Ubiquitin was generously provided by Dr. S. Müller (Max Planck Institute of Biochemistry, Martinsried). All initial plasmids were checked by DNA sequencing at Medigenomix (Martinsried, Germany). For a list of plasmids used in this study see Table 1.

Chemicals and Materials

All chemicals were purchased from Merck, Sigma-Aldrich Chemical Company (Sigma, St. Louis, MO), Fluka-Biochemika (Buchs, Switzerland) or Roth (Karlsruhe, Germany) unless otherwise stated. Components for growth media for *E. coli* were from Difco Laboratories (Lawrence, KS) or Merck (Darmstadt, Germany). The Minigel system was purchased from Bio-Rad., tabletop centrifuges were from Eppendorf.

Antibodies

A Plk4 monoclonal antibody (IgG1) was generated against recombinant MBP-Plk4 (AA715-970) purified from *E. coli*. Anti-c-myc (9E10) (Evan *et al.*, 1985), anti-CP110 (Schmidt *et al.*, 2009a), 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-α-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 AlexaRed-555 and AlexaCy5-647 fluorophores, using the corresponding Antibody Labeling Kits (Invitrogen).

A Plk4 polyclonal antibody was generated against recombinant GST-Plk4 (AA888-970) purified from *E. coli* by Charles River Laboratories (Romans, France). Antibodies were affinity purified using GST-tagged antigen bound to Affigel (Biorad) according to standard protocols after pre-clearing of the serum with Affigel-bound GST.

For a complete list of antibodies used in this study please refer to Table 2.

Cell Culture and Transfections

All cells were grown at 37°C in a 5% CO_2 atmosphere. HeLa, U2OS or HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin (100 µg/ml, Gibco-BRL, Karlsruhe, Germany). Cells adherent on acid treated glass coverslips were transiently transfected using TransIT (Mirus Bio, Madison, WI) according to the manufacturer's protocol. Transient transfections of HEK 293T cells were performed using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) 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 tetracyclin-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^{-1} G418 (Invitrogen) and 50 µg ml^{-1} hygromycin (Merck). U2OS cells were cultured as described previously (Habedanck *et al.,* 2005) and myc-Plk4 expression was induced by the addition of 1 µg ml^{-1} of tetracyclin.

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'). hSas-6 was depleted using the siRNA duplex oligonucleotides previously described (Kleylein-Sohn *et al.,* 2007). βTrCP1 and βTrCP2 were depleted using siRNA duplex oligonucleotides targeting both paralogues (Guardavaccaro *et al.,* 2003). p38α was depleted using two siRNA duplex oligonucleotides (5´-AACTGCGGTTACTTAAACATA-3´; 5´-CTCAGTGATACGTACAGCCAA-3´). Luciferase duplex GL2 was used for control (Elbashir *et al.,* 2001). Transfections were performed using Oligofectamin (Invitrogen) according to manufacturer's protocol. All siRNA duplex oligonucleotides were ordered

from Qiagen, Hilden, Germany. For a complete list of siRNA duplex oligonucleotides used in this study see Table 3.

Cell Extract Preparation and Biochemical Assays

24 hours post transfection, HEK 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, 50 mM NaF, 1 mM PMSF, 25 mM β-glycerophosphate, 1 mM vanadate, Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics)). Lysates were cleared by centrifugation for 15 minutes at 13,000 g, 4°C.

To assay protein degradation kinetics, translation was inhibited by the addition of 25 µg/ml cycloheximide for the indicated time.

For immunoprecipitations, the extracts were incubated with proteinG beads (GE Healthcare) and 10 µg 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 2x 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-βTrCP, which had been *in vitro* translated using the TNT-T7 quick coupled transcription/translation system (Promega) with [^{35}S]-methionine according to the manufacturer's protocol. After washing three times with wash buffer, the bound proteins were eluted by boiling in 2x SDS sample buffer, resolved by SDS-PAGE and analyzed by immunoblotting and autoradiography.

In vitro ubiquitination of *in vitro* translated, [^{35}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 $MgCl_2$, 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 x100/1.4 n.a. oil-immersion objective. Serial optical sections obtained 0.2 μm apart along the Z axis were processed using a deconvolution algorithm and projected into one picture using Softworx.

Name	Tag	Insert	Vector	
pGU173	N-FLAG	β-TrCP1	COM235	pcDNA3.1-N-FLAG
pGU174	3xmyc	β-TrCP1	COM210	pcDNA3.1-3xmyc
pGU177	3xmyc	Plk4-KD-DSG^{AA} (D154A/S285A/289A)	COM210	pcDNA3.1-3xmyc
pGU181	HA	β-TrCP1	COM230	pcDNA3.1-HA
pJW1	N-GFP	Plk4-WT	COM209	pEGFP-C2
pJW2	N-GFP	Plk4-KD D154A	COM209	pEGFP-C2
pJW3	3xmyc	Plk4-KD D154A	COM253	pcDNA3.1-3xmyc-TO
pJW4	3xmyc	Plk4-WT	COM253	pcDNA3.1-3xmyc-TO
pJW14	3xmyc	Plk4-WT AA1-608	COM253	pcDNA3.1-3xmyc-TO
pJW70	3xmyc	Plk4-WT AA609-970	COM253	pcDNA3.1-3xmyc-TO
pJW187	N-FLAG	Plk4-WT-DSG^{AA} (S285A/T289A)	COM263	pcDNA3.1-N-FLAG TO
pJW188	N-FLAG	Plk4-WT-DSG^{AA} (S285D/T289D)	COM263	pcDNA3.1-N-FLAG TO
pRH97	MBP	Plk4-KD	COM226	pMAL-pFN
pRH98	MBP	Plk4-WT	COM226	pMAL-pFN
pRH154	N-FLAG	Plk4-WT	COM263	pcDNA3.1-N-FLAG TO
pRH155	N-FLAG	Plk4-KD	COM263	pcDNA3.1-N-FLAG TO
UK207	HA	Ubiquitin	n/a	Gift from Stefan Müller

Table 1. Plasmids used in this study.

Antigen	Made in	Dilution	Comment	Distributor/Reference
Cep135	rabbit	1:1000	a.p.	Schmidt *et al*., 2007
CP110	rabbit	1:1000	a.p.	Kleylein-Sohn et al., 2007
FLAG	mouse	1:500	a.p.	Sigma
FLAG	rabbit	1:1000	a.p.	Santa Cruz
HA	mouse	1:1000	a.p.	Abnova
myc	goat	1:200	a.p.	Santa Cruz
myc	mouse	1:5	a.p.	Evan *et al*., 1985
myc	rabbit	1:200	a.p.	Santa Cruz
p38α	rabbit	1:1000	a.p.	Cell Signaling
Plk4	mouse	undiluted	hybridoma supernatant	this work
Plk4	rabbit	1:25 - 1:500	a.p.	Kleylein-Sohn *et al*., 2007
αtubulin	mouse	1:1000	a.p.	Sigma

Table 2. Antibodies used in this study.

Gene	Target Sequence (5´→3´)	Reference	oligo #
hSas-6	CTAGATGATGCTACTAAGCAA	Kleylein-Sohn *et al*., 2007	295
Plk4	CTGGTAGTACTAGTTCACCTA	Habedanck *et al.* 2005	302
Plk4 3´-UTR	CTCCTTTCAGACATATAAG	this work	141/142
βTrCP1/2	AAGTGGAATTTGTGGAACATC	Guardavaccaro *et al.* 2003	488
p38α	AACTGCGGTTACTTAAACATA	this work	896
p38α	CTCAGTGATACGTACAGCCAA	this work	897

Table 3. siRNA oligonucleotides duplexes used in this study

Name	Number	Purpose	sequence (5´→3´)
oGU205	M6127	cloning βTrCP	CAAGGATCCAAATGGACCCGGCCGAGG
oGU206	M6128	cloning βTrCP	CAACTCGAGTTATCTGGAGATGTAGGTG
oJW64	M6025	Plk4 mutagenesis S285A / T289A	GAAGACTCAATTGATGCTGGGCATGCCGCAATTTCTACTGC
oJW65	M6026	Plk4 mutagenesis S285A / T289A	GCAGTAGAAATTGCGGCATGCCCAGCATCAATTGAGTCTTC
oJW66	M6027	Plk4 mutagenesis S285D / T289D	GAAGACTCAATTGATGACGGGCATGCCGACATTTCTACTGC
oJW67	M6028	Plk4 mutagenesis S285D / T289D	GCAGTAGAAATGTCGGCATGCCCGTCATCAATTGAGTCTTC

Table 4. Primers used for PCR in this study.

7 ABBREVIATIONS

All units are abbreviated according to the International Unit System.

AA: amino acid(s)
ATP: adenosine 5´-triphosphate
βTrCP: β-transducin repeat containing protein
BSA: bovine serum albumin
Cep: centrosomal protein
CHX: cycloheximide
DAPI: 4´,6-diamidino-2-phenylindole
DTT: dithiothreitol
ECL: enhanced chemiluminescence
EDTA: ethylenedinitrilotetraacetic acid
EGFP: enhanced green fluorescent protein
FCS: fetal calf serum
GFP: green fluorescent protein
HCl: hydrochloric acid
HEPES: N-2-hydroxyethylpiperazine-N`-2-ethane sulfonic acid
IgG: immunoglobulin G
IF: immunofluorescence
IP: immunoprecipitation
IPTG: isopropyl-beta-D-thiogalactopyranoside
mAb: monoclonal antibody
MT: microtubule
MTOC: microtubule-organizing centre
pAb: polyclonal antibody
PCM: pericentriolar material
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
Plk4: Polo-like kinase 4
PMSF: phenylmethylsulfonyl fluoride
RNA: ribonucleic acid
RT: room temperature; reverse transcription
Sak: Snk/Fnk akin kinase
SDS-PAGE: sodium dodecylsulfate polyacrylamid gelelectrophoresis
siRNA: small interference ribonucleic acid
SPB: spindle pole body
ubi: ubiquitin
UTR: untranslated region (of mRNA)
WB: western blot
WT: wild-type

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9 PUBLICATIONS

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10 CURRICULUM VITAE

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