MECHANISMS OF HUMAN PAPILLOMAVIRUS TYPE 16 E7 (HPV-16 E7)-INDUCED DISRUPTION OF CENTRIOLE DUPLICATION CONTROL

by

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Infection with high-risk human papillomaviruses (HPVs) is the main cause of cervical cancer, the second most common cause of cancer-related mortality in women worldwide. High-risk HPV types, such as HPV-16, express two oncoproteins, E6 and E7, which function to subvert critical host cell cycle control mechanisms in order to promote viral genome amplification. Disruption of the pRB signaling axis and the p53-mediated stress response by the HPV E7 and E6 oncoproteins, respectively, results not only in aberrant proliferation but also in host cellular changes that can promote genomic instability. The high-risk HPV-16 E7 oncoprotein was found to induce centrosome abnormalities thereby disrupting mitotic fidelity and increasing the risk for chromosome missegregation and aneuploidy. Aneuploidy is frequently found in pre-malignant high-risk HPV-associated lesions and is a critical factor for malignant progression. This thesis was designed to determine the molecular mechanisms behind the ability of HPV-16 E7 to rapidly induce centriole overduplication. This rapid induction was found to be possible through the simultaneous formation of more than one daughter centriole at single maternal centrioles (centriole multiplication). It was previously discovered that the centriole multiplication pathway relied on cyclin E, CDK2 and PLK4. However, it was not known before how these molecular players cooperate in the centriole multiplication pathway or how HPV-16 E7 expression promotes the activation of this pathway. Here, we report that cyclin E/CDK2 mediates the aberrant recruitment of PLK4 to maternal centrioles. This initial

recruitment step was not sufficient to induce centriole multiplication unless PLK4 protein levels were increased. We found that PLK4 protein levels were controlled by proteolysis, specifically by CUL1-based E3 ubiquitin ligase complexes localized at maternal centrioles. SCF activity was found to control not only baseline PLK4 protein stability but its activity-dependent degradation following cyclin E/CDK2 overexpression. High-risk HPV-16 E7 is known to deregulate cyclin E/CDK2 complexes and we found that ectopic expression of HPV-16 E7 promoted the aberrant recruitment of PLK4 to maternal centrioles. Since our previous experiments have shown that aberrant recruitment of PLK4 is not sufficient to drive centriole overduplication, we determined whether HPV-16 E7 may also disrupt PLK4 expression. We found that HPV-16 E7, but not low-risk HPV proteins or mutants of HPV-16 E7 that lack the ability to induce centriole overduplication, causes a moderate but significant upregulation of PLK4 mRNA. Besides centriole duplication control, we discovered that proteolysis also regulates other aspects of centriole synthesis such as regulation of daughter centriole length. Defining the precise molecular circuitry of centriole biogenesis will aid not only in deepening the current understanding of centriole biogenesis but also aid in identification of novel targets, such as CDK2 or PLK4, for small molecules to prevent centriole abnormalities, mitotic infidelity and malignant progression in pre-invasive high-risk HPV-associated lesions.

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THESIS OUTLINE

Chapter 1 consists of a general introduction to human papillomaviruses and their association with cancer. The role of the high-risk HPV oncoproteins in the viral life cycle and in deregulating centrosome duplication control leading to genomic instability will be discussed. This chapter also provides a review of the centrosome duplication cycle and presents evidence suggesting that HPV-16 E7 oncoprotein disruption of this cycle is a key factor in promoting HPV-associated tumorigenesis.

Chapter 2 describes the Skp1-CUL1-F-box (SCF)-mediated control of centriole biogenesis. CUL1 was shown to localize to centrioles in a cell cycle dependent manner. Knock-down of CUL1 led to centriole overduplication in the form of centriole multiplication. Intriguingly, this centriole multiplication phenotype mimicked that induced by high-risk HPV-16 E7 protein expression. Further experiments revealed that CUL1-mediated restraint of centriole biogenesis involved the degradation of polo-like kinase 4 (PLK4) at maternal centrioles. Additionally, it was shown that CUL1 is critical for the degradation of active PLK4 following deregulation of cyclin E/CDK2 activity as well as baseline PLK4 protein stability. Finally, we discovered that ectopic expression of CAND1, an inhibitor of SCF-ligase complexes, promoted centriole multiplication and stabilized PLK4 protein levels. These results suggest that active CUL1 may

function as a tumor suppressor by regulating PLK4 protein levels and thereby restraining excessive daughter centriole formation at maternal centrioles.

Chapter 3 describes the mechanism by which HPV-16 E7 induces centriole multiplication. We detected aberrant PLK4 protein expression at maternal centrioles in primary human keratinocytes engineered to stably express HPV-16 E7. Real-time quantitative reverse transcriptase (qRT-PCR) revealed an increase in PLK4 transcription in keratinocytes stably expressing HPV-16 E7. The ability of HPV-16 E7 to upregulate PLK4 mRNA was found to be dependent on its ability to degrade the retinoblastoma (pRb) protein and interact with histone deacetylases (HDACs), suggesting a role of E2F-mediated gene transcription in deregulation of PLK4. Collectively, these results highlight the critical role of PLK4 as a regulator of centriole biogenesis and identify PLK4 as a novel target for small molecules to prevent centriole abnormalities, mitotic infidelity and malignant progression in pre-invasive HPV-associated neoplasms.

Chapter 4 describes the role of proteolysis in maintaining daughter centriole length. Here, we show that inhibition of the proteasome by Z-L_3VS or MG132 induces abnormal elongation of daughter centriole microtubules to approximately four times their normal length. Using an siRNA screen, we identified a total of nine gene products that either attenuated (seven) or promoted (two) abnormal Z-L_3VS-induced daughter centriole elongation. Our hits included known regulators of centriole length including CPAP and CP110 but, interestingly, a number of proteins involved in microtubule stability and anchoring as well as centrosome cohesion. These results underscore that daughter centriole length is not limited by structural constraints but is regulated by proteolysis. They furthermore highlight the complexity of daughter centriole length

control and provide a framework for future studies to dissect the molecular details of this process.

Chapter 5 summarizes findings from each of the previous sections and develops a comprehensive model of how the HPV-16 E7 oncoprotein disrupts multiple pathways controlling centriole biogenesis inducing centriole multiplication. This chapter will highlight results which illustrate the importance of proteolysis in controlling centriole duplication processes. Future directions will also be discussed.

1.0 GENERAL INTRODUCTION

1.1 HUMAN PAPILLOMAVIRUSES (HPVS) AND CANCER

Infection with high-risk human papillomaviruses (HPVs) is the main cause of cervical cancer, which is the second most common cause of cancer related mortality in women worldwide [1]. Studies have shown that high-risk HPV DNA is present in >90% of cervical cancers [2]. There are over 100 HPV genotypes, which are classified into two major groups: cutaneous and mucosal. Infection with cutaneous HPV types, for example HPV-1 and -2, usually leads to benign diseases such as skin and plantar warts. However, some cutaneous HPV types, such as HPV-5 and HPV-8, have been associated with skin carcinoma in immunodeficient patients and in patients with the skin disease epidermodysplasia verruciformis (EV) [3, 4]. Mucosal HPVs are further subdivided into low-risk and high-risk HPVs [5]. Low-risk types such as HPV-6 and -11 are associated with benign lesions such as condylomata acuminata and oral and laryngeal papillomas [1]. High-risk HPV types such as HPV-16, -18, -31, -33, and -45 are associated with anogenital and a subset of oropharyngeal tract cancers, in particular cervical carcinoma. Epidemiological and biological studies have shown that HPV-16, and -18 are the most oncogenic types within the high-risk group accounting for 50% and 20%, respectively, of cervical cancers [2].

Despite the high prevalence of HPV infection in sexually active women, most HPV infections are self-limiting and transient. Progression to cancer is a result of both persistent infection with high-risk HPV as well as additional co-factors such as tobacco use, exogenous estrogen and UV-exposure [6]. Mounting evidence suggests that genomic instability may also be an important co-factor in promoting malignant progression. This is supported by evidence suggesting that patients with Fanconia Anemia (FA), a rare X-linked and autosomal recessive chromosomal instability syndrome, are at a significantly higher risk for developing HPV-

associated cancerous lesions [7-9]. In addition, genomic instability is a frequent and early event during HPV-associated malignant progression and detected in a significant fraction of pre-invasive high-risk HPV-associated lesions [10, 11]. Importantly, there is evidence suggesting that HPV oncoproteins can by themselves drive genomic instability [12, 13]. Together, these observations lend support to the idea that genomic instability is a critical factor for malignant progression of high-risk HPV-associated cancers.

Recently, prophylactic vaccines have been developed against HPV-6, -11, -16, and -18 and a small but growing proportion of the world-wide population is being vaccinated to prevent HPV infection [14]. However, the vaccines are currently still expensive and prevention of HPV-associated carcinoma is only effective in people with no prior exposure to high-risk HPV. Understanding the exact mechanisms by which high-risk HPV oncoproteins promote chromosomal instability will not only provide novel insights into basic biological processes but may also contribute to the development of better preventive and therapeutic options complimentary to preventative vaccination.

1.2 GENERAL BIOLOGY OF HPVs

High-risk HPVs, such as HPV-16, are circular double-stranded DNA viruses of approximately 8,000 base pairs. Oncogenic HPV genomes contain eight open reading frames (ORFs) which are expressed as polycistronic mRNAs in a temporal manner under control of the non-coding long control region (LCR) (Fig. 1). The LCR contains the viral origin of DNA replication and important transcriptional control elements recognized by both cellular and virally

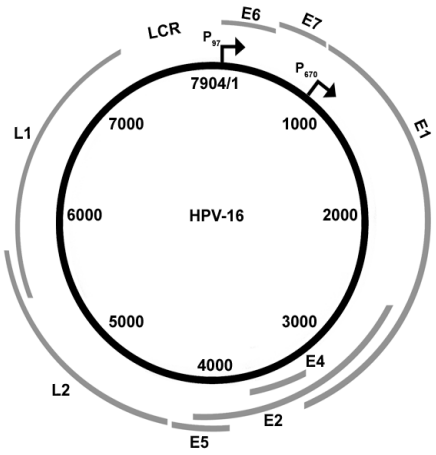

Figure 1. The HPV-16 Genome.

HPV-16 contains eight open reading frames (ORFs) under the transcriptional control of the non-coding long control region (LCR). HPV early (E) genes control the viral lifecycle and late (L) genes encode viral structural proteins. HPV-16 contains two major promoters an early promoter (P_{97}) and a late promoter (P_{670}). The early promoter is upstream of the E6 ORF and is active early following infection, while the late promoter is found within the E7 ORF and is activated upon differentiation during the productive phase of the viral lifecycle

encoded regulatory proteins [15]. HPV (E) early transcripts control viral gene transcription and deregulate host targets to allow amplification of the viral genome in terminally growth-arrested cells. Early transcripts of high-risk HPV-16 are under control of a single promoter P_{97}, contained within the LCR [15]. HPV late (L) transcripts, L1 and L2, encode for the major and minor viral capsid proteins, respectively. Late transcripts of high-risk HPV-16 are under control of the late promoter P_{670}, residing within the E7 coding region, which only becomes active in terminally differentiating keratinocytes [16].

The HPV lifecycle is intimately linked to the differentiation state of host keratinocyte cells [15]. HPVs are thought to infect cells of the basal layer of the epithelium through microabrasions to the epithelium surface. The basal strata contain stems cells and transit-

amplifying cells which undergo cell division in order to maintain the suprabasal layer [15]. As progeny cells of the basal epithelium layer migrate towards the upper stratum they normally undergo terminal differentiation and permanently exit the cell cycle. HPV infected cells remain capable of cell cycle progression through the actions of the HPV oncoproteins [15].

Upon HPV infection, E1 and E2 gene expression is activated. The E1 protein functions as a DNA helicase and interacts with the E2 protein to bind to the viral origin of replication [17-19]. E2 also acts as a transcriptional regulator both activating and limiting the expression of the viral genes E6 and E7 during the early phase of virus infection. The virus does not encode any of its own replicative enzymes and is entirely dependent on host cell DNA replication machinery. As viral replication occurs in differentiating keratinocytes that have permanently withdrawn from the cell division cycle, the E6 and E7 oncoproteins have evolved to ensure the formation of an S-phase milieu which is necessary for replication of the viral genome [20].

The E4 and E5 proteins play a less well understood role in the viral lifecycle. The E4 protein may play a role in virus egress from the cell by inducing the collapse of the cytokeratin network [21]. The E5 protein is necessary for optimal growth of the virus, possibly involving interaction with the epidermal growth factor receptor (EGFR) [22, 23].

During the productive phase of the viral lifecycle, the HPV genomes replicate on average once per cell cycle during S-phase, in synchrony with host cell DNA replication [15]. The HPV genome is normally maintained episomally, at approximately 50-100 copies per cell [24]. Once an infected cell reaches the upper epidermal layers and undergoes terminal differentiation, a burst of viral DNA replication occurs, producing the viral genomes to be packaged into progeny virions [15].

1.3 HIGH RISK HPV ONCOPROTEINS

High-risk HPVs express two oncoproteins, E6 and E7, which function to de-regulate the host cell cycle in order to promote amplification of the viral genome. Long-term expression of HPV E6 and E7 oncoproteins are known to both extend the life-span of primary human cells and facilitate their immortalization [25]. High-risk HPV E6 and E7 gene expression is consistently up-regulated in HPV-associated malignant tumors, in which viral genomes are frequently found integrated into host cell chromosomes [26].

Integration of the viral genome terminates the productive lifecycle of the virus. Viral genome integration can occur throughout the host genome, but most frequently is found at common fragile sites [27]. Integration most commonly results in deletion of a large segment of the viral genome, while the E6 and E7 ORFs remain intact along with the LCR, which lies upstream of the integration site within the viral genome [26]. The E1 and E2 viral genes are disrupted, resulting in higher levels of both E6 and E7 gene transcription.

However, overexpresion of the HPV-16 E6 and E7 genes is not necessary for induction of genomic instability, suggesting that the high-risk HPV E6 and E7 oncoproteins promote an increased risk of malignant progression even when their expression is tightly controlled [28].

1.3.1 The HPV-16 E7 oncoprotein

High-risk HPV E7 proteins are small phosphoproteins with no known human homologs [29]. HPV E7 oncoproteins contain two conserved domains (CR1 and CR2) which share sequence similarity to both adenovirus E1A and SV40 large T antigen (Fig. 2) [30, 31]. High-risk HPV E7 inactivates the retinoblastoma tumor suppressor protein (pRB), and the related

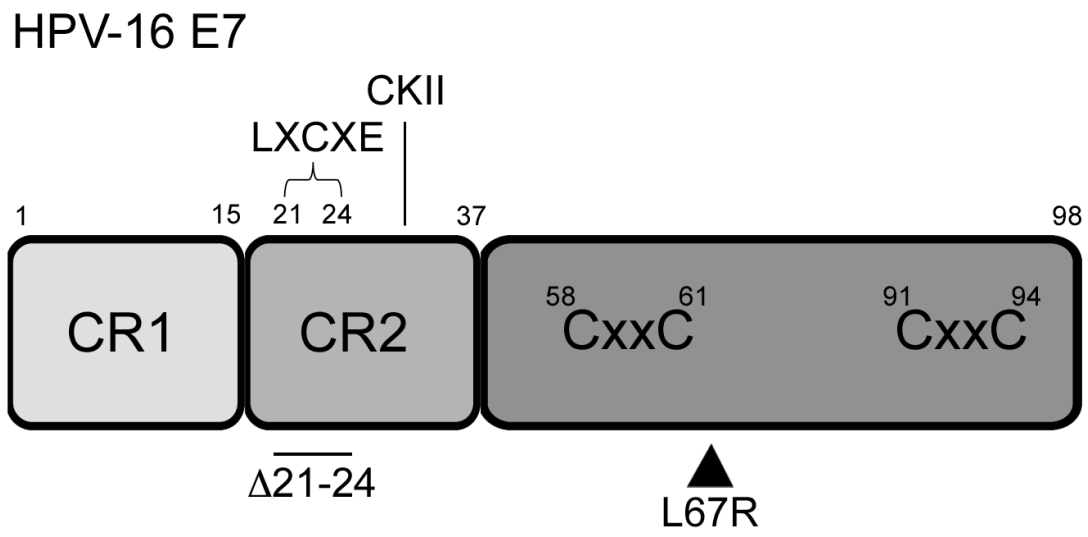

Figure 2. Schematic of the HPV-16 E7 oncoprotein.

The HPV-16 E7 oncoprotein is a small 98 amino acid protein containing two conserved domains, CR1 and CR2, and a carboxy-terminal region characterized by two metal-binding regions. The CR2 domain contains the LXCXE pRB-binding domain and a caesin kinase II phosphorylation motif. The HPV-16 E7 mutant with deletion of amino acids 21-24 (Δ21-24) is unable to bind and degrade pRB. The HPV-16 E7 mutant with amino acid substitution at L67 (L67R) is unable to interact with HDACs and has a decreased ability to transactivate gene transcription.

pocket protein family members p107 and p130, which are responsible for regulating E2F-mediated transcription of S-phase genes [32-34]. Specifically, HPV-16 E7 binds to and induces the proteasomal degradation of pRB by cullin 2-containing E3 ubiquitin ligases [34-36]. High-risk HPV E7 associates with pRB and its family members through a Leu-X-Cys-X-Glu (LXCXE) motif located within the CR2 homology domain [37]. Additional sequences located in the amino-terminal CR1 homology domain are necessary for pRB degradation [35]. High-risk HPV-16 E7 has also been shown to inactivate p600, a pRB-associated protein [38, 39].

An important functional difference between low-risk and high-risk HPV E7 oncoproteins lies in their ability to bind and degrade pRB, p107 and p130. High-risk HPV-16 E7 binds with a

higher affinity to pRB-family members than do low-risk HPV-6 E7 proteins. In the case of pRB, this difference maps to a single amino acid change within the pRB-binding domain which confers high-affinity binding [40]. Low-risk HPV-6 also binds p107 and p130 with a lower affinity however this difference does not map to the same residue as pRB-binding efficiency [41]. Moreover, HPV-6 E7 has recently been shown to destabilize p130, but not pRB or p107, suggesting that disruption of signaling pathways controlled by p130 are necessary for the productive stage of the viral lifecycle and that pRB and/or p107 degradation are important for carcinogenesis [41].

To further disrupt host gene expression control, HPV-16 and HPV-31 E7 oncoproteins also interact with histone deacetylases type -1 and -2 (HDAC-1, and -2) [42, 43]. HDACs function as transcriptional repressors by reversing acetyl modifications of lysine residues on histones. The indirect association between oncogenic HPV-16 E7 and HDACs is mediated by Mi2β, a component of the NURD histone deacetylase complex [42]. This interaction is dependent on the integrity of two Cys-X-X-Cys motifs in the HPV E7 oncoprotein carboxy-terminus and results in increased E2F-mediated gene transcription from HDAC responsive promoters [42]. High-risk HPV-16 E7 also associates with histone acetyl transferases (HATs) such as, p300 and pCAF, which function to activate transcription and stimulate cellular proliferation [44, 45].

In addition to chromatin remodeling, high-risk HPV-16 E7 can directly alter cellular transcription through interaction with E2F1. This interaction results in the pRB-independent enhancement of E2F-mediated gene transcription [46]. However, the promoter of E2F6, a transcriptional repressor responsible for directing cell cycle exit, is also E2F-responsive [47]. The HPV-16 E7 oncoprotein has therefore evolved to directly associate with E2F6 resulting in

inactivation of its transcriptional repression and maintenance of an S-phase like environment [48].

Together, along with the ability of HPV-16 E7 to interact with cyclin/CDK complexes and its ability to overcome cellular growth arrest signals mediated by the cyclin dependent kinase (CDK) inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, the HPV E7 oncoprotein profoundly disrupts the pRB-signaling axis to favor replication of the viral genome [49-51]. Why high-risk HPV E7 has evolved to target a multitude of G_1/S checkpoint components to achieve this goal is currently unknown.

1.3.2 The HPV-16 E6 oncoprotein

Disruption of the host cell cycle by HPV-16 E7 is likely to activate cellular stress responses and apoptotic signaling cascades. The HPV E6 oncoprotein has evolved to inhibit the host cell response to unscheduled cell cycle entry by mediating the degradation of p53. High-risk E6 degrades p53 by re-directing a host cell HECT domain containing E3 ubiquitin ligase, E6-associated protein (E6AP) [52]. Moreover, HPV-16 E6 binds the transcriptional co-activators CBP/p300 and decreases the ability to activate p53-responsive promoter elements [53]. High-risk HPV E6 has been suggested to switch p53-p300 from an activating to a repressor complex independently of E6AP [54].

High-risk HPV E6 has additional p53-independent functions that are important for cellular transformation and immortalization. Oncogenic HPV E6 contains a PDZ-domain binding motif, X-(S/T)-X-(V/I/L)-COOH, that is unique to high-risk HPV E6 and is not present in low-risk HPV E6 [55]. The HPV E6 oncoprotein binds PDZ-containing host proteins targeting them for degradation in both an E6AP-dependent and -independent manner [56-58]. Candidate PDZ-

containing protein targets include hDlg, hScrib, MAG1-3, and MUPP1 [56, 57]. PDZ-containing proteins localize to membrane-cytoskeleton interfaces and have been implicated as molecular signaling scaffolds modulating cell growth, polarity and adhesion in response to cell contact. The targeted inactivation of these proteins by oncogenic HPV E6 may disrupt cell junctions, induce loss of cell polarity and promote cellular transformation [59].

The oncogenic HPV-16 E6 protein promotes cellular immortalization through the transcriptional up-regulation of hTERT, the catalytic subunit of human telomerase, and can contribute to telomere maintenance [60]. High-risk HPV E6 can enhance hTERT transcription through several mechanisms including association with the transcriptional activator c-Myc and/or the E6AP-dependent degradation of a putative transcriptional repressor of the hTERT promoter, NFX1-91 as well as others [61, 62].

1.4 HIGH RISK HPV ONCOPROTEINS AND GENOMIC INSTABILITY

Genomic instability is a defining phenotype of many malignant tumors including HPV-associated malignancies [63, 64]. Over 100 years ago, Theodor Boveri hypothesized that genomic instability and cancer can result from the presence of extra centrosomes and the subsequent formation of multipolar mitoses. Such a disruption of spindle polarity may consequently promote chromosome missegregation and ultimately aneuploidy [65]. The major consequences of supernumerary centrosomes are polarity disturbances, such as multipolar mitoses, and/or merotelic kinetochore attachments, which can lead to lagging chromosomes during cell division [66]. However, extra centrosomes do not necessarily lead to cell division errors since centrosomes can cluster thereby preserving bipolarity of the mitotic spindle [67].

Nonetheless, centrosome abnormalities have been detected in a wide range of malignant tumors including breast, prostate, colon and cervical cancer, and compelling evidence suggests that centrosome abnormalities can drive progressive loss of genomic stability and malignant progression [63, 68].

Studies in HPV-associated primary anal human tumors have demonstrated that centrosome overduplication correlates with the frequency of cell division errors, which lends important support to the notion that the presence of overduplicated centrosomes can promote these defects in HPV-associated carcinomas [69]. In line with this finding, the frequency of aneuploidy increases with both malignant grade and tumor aggressiveness in HPV-associated lesions [70, 71]. Correlating with the increase in aneuploidy, multipolar, specifically tri-polar, mitoses are a hallmark of high-risk HPV-associated carcinomas [72].

In the context of high-risk HPV, centrosome amplification is observed in cells expressing episomal HPV-16 genomes, which underscores that viral integration and overexpression of HPV E6 and HPV E7 oncoproteins is not required for the disruption of centrosome duplication control [28]. HPV-16 E7 expression directly disrupts centriole duplication control resulting in the rapid induction of centriole overduplication, whereas HPV-16 E6 expression promotes the accumulation of centrioles in cells that are already genomically unstable [72]. Determining the pathways that are activated by HPV oncoprotein expression leading to centrosome overduplication, cell division errors and ultimately aneuploidy will be important in understanding and preventing the earliest steps in malignant progression.

1.5 THE CENTROSOME

The centrosome is the major microtubule-organizing center of most mammalian cells and orchestrates bipolar spindle pole formation during mitosis [73]. The centrosome consists of two centrioles embedded in a cloud of pericentriolar proteins, also known as pericentriolar material (PCM) [74]. The two centrioles differ in age and composition, consisting of an older maternal centriole characterized by distal and sub-distal appendage proteins which function to anchor and nucleate microtubules, and a younger daughter centriole which does not yet associate with the appendage proteins [73]. In order to generate two spindle poles, the single centrosome of a non-dividing cell must duplicate precisely once, and only once, prior to mitoses in order to ensure faithful cell division.

1.5.1 Centrosome duplication

Centrosome duplication begins during late mitosis/early G_1-phase of the cell division cycle, when the two pre-existing centrioles of the single centrosome disengage through the action of polo kinase 1 (PLK1) and separase, and move into a near parallel position (Fig. 3) [75]. This step is followed by recruitment of polo-like kinase 4 (PLK4) to the wall of the maternal centriole at the site of daughter centriole synthesis [76]. Subsequently, structural proteins are recruited to the nascent pro-centriole to stabilize and elongate the new daughter centriole. Centrosome duplication is complete during late G_2-phase of the cell cycle, when the two fully formed centriole pairs separate to form the mitotic spindle poles [73].

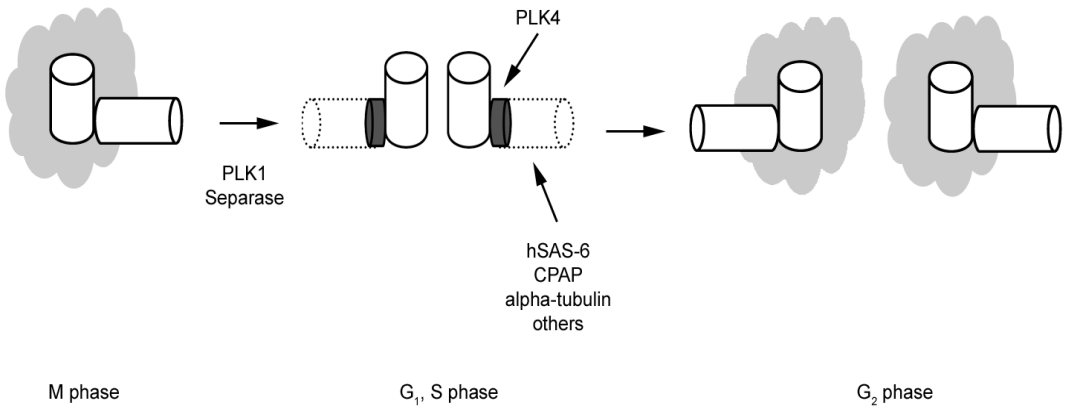

Figure 3. The centrosome duplication cycle.

Centrosome duplication occurs in synchrony with the cell division cycle beginning with separation of the two centrioles through the actions of PLK1 and separase. This step is followed by recruitment of PLK4, to the wall of the pre-existing, or maternal centrioles, at the site of daughter centriole synthesis. Each maternal centriole serves as a platform for the synthesis of exactly one daughter centriole. Centriolar structural proteins such as hSAS-6, CPAP, alpha-tubulin and others are recruited to stabilize and elongate the daughter centriole. Centrosome duplication is complete during the late-G_2 phase of the cell cycle, when the two fully formed centriole pairs separate to form the mitotic spindle poles.

1.5.2 Mechanisms of centriole amplification

There are two mechanisms by which centriole amplification in tumor cells may occur: centriole overduplication and centriole accumulation (Figure 4). These two phenotypes can be distinguished by immunostaining for markers of older, mature centrioles [77]. Genuine centriole overduplication is characterized by the presence of one or two mature maternal centrioles and multiple immature daughter centrioles. In contrast, centriole accumulation is defined by the presence of multiple maternal centrioles with a normal ratio of daughter centrioles (Fig. 4) [77].

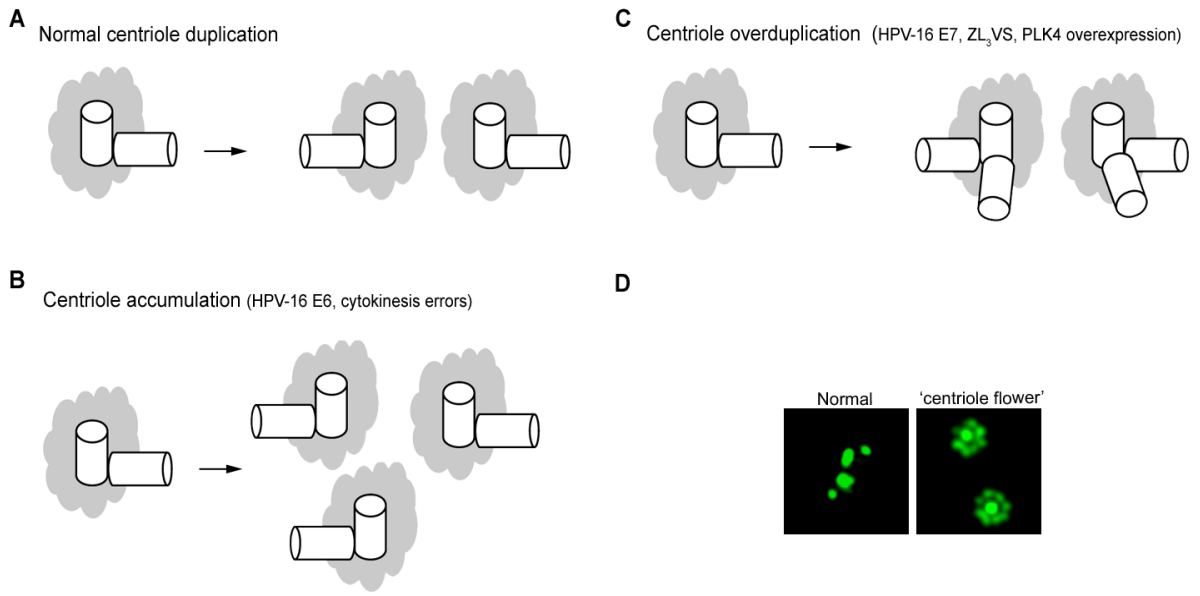

Figure 4. Schematic of centriole amplification phenotypes.

(A) Following normal centriole duplication one or two maternal centrioles associate with a single daughter centriole to form two functional centrosomes (B) Centriole accumulation occurs due to cytokinesis or other cell division errors and results in cells containing more than two maternal centrioles, each associated with a single daughter centriole. (C) Centriole overduplication arises due to direct disruption of the centriole duplication cycle and is characterized by one or two maternal centrioles in the presence of multiple daughter centrioles. (D) Fluorescence microscopic analysis of normal duplicated centrioles and 'centriole flower' phenotype induced by PLK4 overexpression in U-2 OS/centrin-GFP cells.

The distinction between centriole overduplication and accumulation is important because cells exhibiting centriole accumulation may arise due to abortive mitoses or cytokinesis errors and such cells may not be able to produce viable progeny. Conversely, cells which exhibit a genuine centriole overduplication defect are, in general, less genomically altered and hence are more likely to give rise to genomically unstable daughter cells.

In vitro studies have demonstrated that the HPV-16 E7 oncoprotein, in particular, disrupts genomic integrity by directly interfering with centriole duplication control. High-risk HPV-16 E7

expression produces abnormal centriole numbers in otherwise normal cells prior to the onset of genomic instability [78]. In contrast, high-risk HPV-16 E6 expressing cells exhibit centrosome accumulation in cells which are already genomically unstable, often expressing markers of cellular senescence, and are unlikely to remain in the proliferative pool (see section 1.7) [78].

1.6 IN VIVO MODELS OF HPV ONCOPROTEIN INDUCED CENTROSOME ABNORMALITIES AND MALIGNANT PROGRESSION

The *in vivo* role of HPV-16 E7-induced centrosome abnormalities in malignant progression is highlighted in a transgenic mouse model of cervical carcinogenesis. Transgenic mice expressing HPV-16 E7 driven by a cytokeratin 14 promoter and treated with low doses of estrogen develop numerical centrosome abnormalities in the cervical mucosa which progress to invasive carcinomas [79]. In contrast, HPV-16 E6 expressing transgenic mice display a comparable level of numerical centrosome aberrations but develop only low grade cervical lesions that do not progress to malignant tumors [79]. These results suggest that centrosome aberrations in the context of HPV-16 E7 expression are associated with a greater risk of malignant progression than in HPV-16 E6 expressing cells. Importantly, this underscores that centrosome abnormalities that arise in the context of high-risk HPV-16 E6 expression may not necessarily contribute to carcinogenesis.

1.7 HPV-16 E7 ONCOPROTEIN AND DISRUPTION OF CENTROSOME DUPLICATION CONTROL

Following high-risk HPV-16 E7 expression, supernumerary centrioles appear rapidly and within a single cell division cycle, suggesting they arise due to direct disruption of centriole duplication control [80]. This was initially difficult to reconcile with the prevailing model of centriole duplication described above, where a single maternal centriole initiates the synthesis of only a single daughter centriole. Further analysis of HPV-16 E7 induced centriole abnormalities led to the discovery that the HPV E7 oncoprotein rapidly induces centriole overduplication through stimulation of a novel centriole duplication pathway, referred to as centriole multiplication [80]. This pathway is characterized by a single maternal centriole initiating the simultaneous synthesis of two or more daughter centrioles (Fig. 4C). Centriole multiplication had not previously been observed in the context of an oncogenic stimulus relevant for a major human cancer [80].

1.7.1 Additional mechanisms of HPV-16 E7-induced centrosome abnormalities

An HPV-16 E7 mutant with deletion of the amino acid region 21-24 which contains the LCXCE motif (HPV-16 E7 Δ21-24, see Figure 2) was found to be unable to induce centriole overduplication in both normal and pRB-family deficient mouse embryo fibroblasts [81]. In contrast, full-length wild-type HPV-16 E7 was able to induce centriole abnormalities even in pRB/p107/p130-deficient cells [81]. These results suggest that the oncogenic HPV E7 protein induces centriole overduplication in both pRB-dependent and -independent mechanisms. One possible mechanism for pRB-independent induction of supernumerary centrioles is through the

ability of HPV-16 E7 to interact with γ-tubulin, a component of the PCM important for microtubule nucleation. This interaction, which is pRB-independent, relies on an intact LXCXE motif [82]. It has been suggested that disruption of γ-tubulin plays a role in the regulation of centrosome duplication and this mechanism may hence contribute to overduplication induced by HPV-16 E7 [74].

Other viral oncoproteins, such as Adenovirus E1A and the HTLV-1 Tax protein have also been found to promote centriole overduplication and genomic instability although the underlying molecular mechanism appears not to be uniform [83, 84].

1.8 THE CENTRIOLE MULTIPLICATION PATHWAY

Multi-ciliated epithelial cells such as those in the trachea and oviduct can rapidly produce hundreds of centrioles during ciliogenesis through the centriole multiplication pathway [85, 86]. Besides expression of the high-risk HPV-16 E7 protein, aberrant induction of the centriole multiplication pathway can be stimulated in other cell types by overexpression of the centriolar structural component hSAS-6 or the protein kinase PLK4 [76, 87, 88]. Despite identification of these molecular players it is currently unknown if the HPV-16 E7 oncoprotein induces centriole multiplication through deregulation of these proteins or through unidentified regulators of this pathway.

Additional insights that may help to determine how the HPV-16 E7 oncoprotein promotes centriole multiplication were recently gained through studies utilizing the proteasome inhibitor Z-L_3VS [80]. When U-2 OS cells were treated with Z-L_3VS centriole multiplication was strongly induced with the formation of so-called ‘centriole flowers’ (Figure 4D). Centriole flowers are

maternal centrioles which associate with the maximum number of possible daughter centrioles [76, 80]. This phenotype was dependent on CDK2, cyclin E and PLK4 [80]. These findings led to the hypothesis that centriole biogenesis is normally restrained by proteolysis. Collectively, these findings suggest that the high-risk HPV-16 E7 protein may interfere with either the regulation or activity of CDK2, cyclin E and PLK4 and/or the proteolytic control of this pathway in order to induce centriole multiplication.

1.9 CELLULAR PROTEOLYSIS AND THE PROTEOLYTIC CONROL OF CENTRIOLE BIOGENESIS

A substrate is targeted for degradation by the proteasome through addition of a polyubiquitin chain to the substrate. This reaction is catalyzed by a three-step enzymatic cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin targeting enzyme (E2), and a ubiquitin-ligase protein (E3). The ubiquitin E3 ligase confers substrate specificity to the reaction [89]. Cullin-RING ubiquitin-ligases (CRLs) are members of the largest family of eukaryotic E3 ligases [90]. Because CRLs are active during late G_1 until early mitosis during the onset of centriole duplication, proteasome inhibition strongly induces centriole multiplication, and several components of CRLs have been localized to the centrosome, CRL activity may be important in restraining centriole biogenesis [91-93].

The CRLs are multisubunit E3 ubiquitin ligase complexes characterized by a common cullin-containing scaffold. Human cells express seven cullin subunits (CUL1, -2, -3, -4A, -4B, -5, and -7) responsible for nucleating the assembly of unique ubiquitin ligase complexes [90]. All CRLs consist of a cullin-backbone, a zinc-binding RING-domain containing protein which

recruits the ubiquitin-conjugating E2 enzyme, and an adaptor protein which recruits interchangeable substrate recognition subunits. In the case of the prototypical CRL, the SKP1-CUL1-F-box (SCF) ubiquitin ligase complex, SKP1 acts as the adaptor protein interacting with substrate recognition subunits containing an F-box domain and a second protein-protein interaction domain which recognizes the specific target protein (Fig. 5) [90]. Other CRLs contain unique adaptor proteins which recognize substrate recognition subunits with different functional motifs [90].

CRL activation is a complex process involving several regulatory pathways and conjugation of a ubiquitin-like protein to the cullin core component [94]. Addition of Nedd8 (neddylation) to a carboxy-terminal lysine residue conserved in all cullin subunits enhances the activation of the ubiquitin-ligase complex [95]. Removal of Nedd8 (deneddylation) is accomplished by the COP9 signalosome in response to depletion of the target substrate [96]. A further regulatory protein, CAND1, binds unneddylated cullin-backbones to prevent the inappropriate formation and activation of CRL complexes [97].

In line with the hypothesis that CRL complexes may restrain centriole biogenesis, studies performed in *Drosophila melanogaster* cells demonstrated that PLK4 protein levels were controlled by an SCF ubiquitin ligase complex containing the F-box protein Slimb [98, 99]. Knock-down of Slimb resulted in PLK4 accumulation and centriole multiplication. The mechanism of SCF ligase-mediated regulation of centriole biogenesis in particular the role of β-TRCP, the mammalian homolog of Slimb, in human cells has not been studied in detail.

The first part of this study reports that SCF E3 ligase activity restrains centriole biogenesis through regulation of PLK4 protein level in human cells. We also identify a mechanism for PLK4 recruitment to maternal centrioles involving the activity of cyclin E/CDK2 complexes and demonstrate that PLK4 protein level is the rate-limiting step in the centriole multiplication pathway.

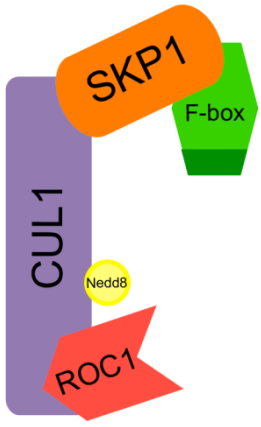

Figure 5. Schematic of Skp1-CUL1-F-box (SCF) ubiquitin ligase complexes.

The SCF ligase, prototypical member of the cullin-RING E3 ubiquitin ligase family, consists of a CUL1-backbone (purple), a carboxy-terminal RING-domain containing protein (Roc1, red), an amino-terminal adaptor protein (SKP1, orange), and an F-box containing substrate specificity subunit (light green) with a second protein-protein interaction domain with recognizes the target substrate (dark green). SCF activity is enhanced by the conjugation of Nedd8 (yellow), a ubiquitin-like molecule, to the cullin backbone.

2.0 THE SKP1-CULLIN-F-BOX (SCF) COMPONENT CUL1 IS A CENTROSOMAL SUPPRESSOR OF CENTRIOLE MULTIPLICATION

Work described in this section was published in *Cancer Research* (*Cancer Res.* 2009, 69:6668-6675) with authors Nina Korzeniewski, Leon Zheng, Rolando Cuevas, Joshua Parry, Payel Chatterjee, Brittany Anderton, Anette Duensing, Karl Münger and Stefan Duensing

L. Zheng and B. Anderton performed preliminary immunofluorescence and knock-down analysis. P. Chatterjee, J. Parry, and R. Cuevas performed several of the western blot experiments. N. Korzeniewski performed all other experiments described in this section. A. Duensing helped with the experimental design and data analysis. K. Münger and S. Duensing conceived the project. N. Korzeniewski and S. Duensing analyzed the results and wrote the manuscript.

Work described in this section on CAND1 is in preparation for submission to *Molecular Cancer* with authors Nina Korzeniewski, Anette Duensing and Stefan Duensing.

N. Korzeniewski performed all of the CAND1 experiments. A. Duensing helped with experimental design and data analysis. N. Korzeniewski and S. Duensing analyzed the results and wrote the manuscript.

2.1 INTRODUCTION

As reviewed in Chapter 1, the single centrosome of a non-dividing cell must duplicate once prior to mitosis in order to organize a bipolar mitotic spindle. Centrosome duplication is a process which is frequently disrupted in tumor cells, where centrosome overduplication has been implicated in mitotic defects, chromosome missegregation and aneuploidy [100-103].

During a normal centrosome duplication cycle, a single maternal centriole generates one and only one daughter centriole. However, recent studies have revealed that proteasomal inhibition induces the single maternal centriole to mediate the simultaneous formation of multiple daughter centrioles (centriole multiplication) [80]. Given the intrinsic potential of maternal centrioles to give birth to multiple daughters, the question arises what the molecular mechanisms that normally restrain procentriole formation to only one per cell division cycle may be.

As detailed in the previous chapter, the centrosome has been shown to harbor components of the ubiquitin-proteasome machinery including subunits of SCF E3 ubiquitin ligases and components of the 26S proteasome [104-107]. Although SCF complexes have been implicated in centriole duplication control as well as centriole separation, their precise functions have not been fully delineated [92].

In general, activation of SCF complexes occurs when Nedd8, a ubiquitin-like protein, is conjugated (neddylated) on a specific lysine residue located in the carboxy-terminal domain of the cullin backbone [108]. This is accomplished by Nedd8 specific E1 and E2 enzymes and Roc1 [109, 110]. Removal of Nedd8 (deneddylation), and therefore SCF inactivation, is accomplished by the COP9 signalosome complex, or CSN (reviewed in [111]).

A further mechanism to prevent inappropriate SCF activation is through binding to CAND1, a cellular regulatory protein which associates exclusively with deneddylated cullin subunits. CAND1 binding to the cullin backbone prevents access of SKP1-F-box protein complexes to the cullin core and therefore inhibits formation of the SCF complex [97, 112]. Neddylation of CUL1, coupled with substrate availability and formation of the SKP1-F-box protein-substrate complex, dissociates CAND1 allowing activation of the SCF-complex to occur [113].

Here, we show that the SCF core component, CUL1, localizes to maternal centrioles and that these centrioles serve as assembly platforms for oncogene-induced centriole overduplication. Moreover, SCF ubiquitin ligase activity was found to be critically involved in suppressing centriole multiplication in human tumor cells by regulating PLK4 protein levels. We provide evidence that SCF ubiquitin ligase activity is critical for the activation-dependent degradation of PLK4 following overexpression of cyclin E/CDK2, as well as for baseline PLK4 protein stability. Furthermore, we show that CAND1, an inhibitor of SCF ligase activity, also localizes to maternal centrioles. Overexpression of CAND1 enhanced the ability of PLK4 to induce centriole multiplication and stabilized PLK4 protein levels. Collectively, our results suggest that SCF ubiquitin ligase activity provides an important mechanism for restraining excessive daughter centriole formation at single maternal centrioles and hence centrosome-mediated cell division errors and chromosomal instability. Since HPV-16 E7 expression induces centriole multiplication in a phenotype reminiscent of CUL1 inactivation, this study provides a framework in which to study the molecular mechanism behind HPV-16 E7-induced centriole multiplication.

2.2 MATERIALS AND METHODS

Cell culture, transfections and inhibitor treatments

U-2 OS or U-2 OS/centrin-GFP cells (centrin-GFP plasmid was kindly provided by Michel Bornens, Institut Curie, France [114]) were maintained as previously reported [80]. For transient transfections (48 h), pCMV- or pcDNA3-based plasmids encoding c-MYC (kindly provided by Philip Leder, Harvard Medical School, Boston, MA), HPV-16 E7, E2F-1 (kindly provided by Jacqueline Lees, Massachusetts Institute of Technology, Boston), cyclin E (provided by Robert Weinberg through Addgene, Cambridge, MA, USA [115], CDK2 (provided by Sander van den Heuvel through Addgene [116], Myc-PLK4 or catalytically inactive Myc-PLK4-D154A (kindly provided by Erich A. Nigg (Max Planck Institute of Biochemistry, Martinsried, Germany [76]), MYC-CAND1, HA-CUL1, and CUL1 mutant plasmids CUL1-RL, CUL1-DN53, CUL1-DC22 (kindly provided by Yue Xiong, University of North Carolina, Chapel Hill [112]), dominant-negative CUL1 (DN-CUL1; provided by Wade Harper through Addgene [117]) or empty vector controls were used and transfected by lipofection (Fugene 6; Roche). Cells were co-transfected with a vector encoding red fluorescent protein targeted to mitochondria (DsRED; BD Biosciences Clontech, Palo Alto, CA) as transfection marker. Cells were treated with 1 μM of the proteasome inhibitor Z-L_3VS (Biomol, Plymoth Meeting, PA) or 0.1% DMSO as control. To inhibit CDK activity, cells were treated with 1 μM of indirubin-3'-oxime (IO; kindly provided by Laurent Meijer, Station Biologique Roscoff, France) or 0.1% DMSO as control. Cycloheximide (CHX; Calbiochem, San Diego, CA) was used at 30 μg/ml for the indicated time intervals with dH_2O as control.

Immunological Methods

Immunoblotting was performed as described previously [80]. Primary antibodies used for immunoblotting were directed against CUL1, cyclin E, CDK2, CAND1 (all Santa Cruz, Santa Cruz, CA), Myc-tag, (Cell Signaling, Danvers, MA), OctA-Probe/Flag® (Santa Cruz) or actin (Sigma, St. Louis, MO).

Immunofluorescence stainings using CUL1 (Neomarkers, Fremont, CA), γ-tubulin (Sigma), CEP170 (Invitrogen, Carlsbad, CA) or PLK4 (mouse monoclonal antibody kindly provided by Erich A. Nigg, Max Planck Institute of Biochemistry, Martinsried, Germany) antibodies were performed as described previously [80]. Primary antibodies were detected with FITC-, Rhodamine Red- or AMCA-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) as previously described [80].

Small interfering RNA (siRNA)

Synthetic RNA duplexes to reduce CUL1 expression were obtained commercially (Ambion, Austin, TX) and used according to manufacturer's protocol.

Statistical Methods

Student's t test for independent samples was used wherever applicable.

2.3 RESULTS

CUL1-positive maternal centrioles are assembly platforms for oncogene-induced centriole overduplication

To explore the role of SCF ubiquitin ligase activity in centriole biogenesis, an immunofluorescence microscopic analysis of the SCF core component CUL1 was performed using U-2 OS cells stably expressing centrin-GFP. CUL1 was found to co-localize with the centriolar maker centrin (Fig. 6A). The co-localization pattern of CUL1 with centrin-GFP suggested that CUL1 may be present at older, mature centrioles (Fig. 6A, middle and bottom panels). A co-immunofluorescence microscopic analysis of CUL1 and CEP170, a maker for mature maternal centrioles [77], in U-2 OS/centrin-GFP cells revealed that CUL1 in fact localizes to older, maternal centrioles (Fig. 6B).

Overexpression of the HPV-16 E7 oncoprotein has previously been shown to lead to excessive daughter centriole formation at maternal centrioles [80, 118]. U-2 OS/centrin-GFP cells were transiently transfected with HPV-16 E7, or other oncogenes including c-MYC oncogene or the transcription factor E2F-1 followed by immunofluorescence staining for CUL1. Since normal centriole duplication generates a maximum of two mature maternal centrioles and a maximum of two immature daughters, excessive numbers of CUL1-negative centrioles in the presence of one or two CUL1-positive centrioles were counted as abnormal. An increase of cells with more than 4 centrioles in the presence of only one or two CUL1-positive centrioles was detected in cells overexpressing c-MYC (6.7%), HPV-16 E7 (10.7%, $p \leq 0.05$) or E2F-1 (13%, $p \leq 0.005$) in comparison to empty vector control (3%) (Fig. 6C).

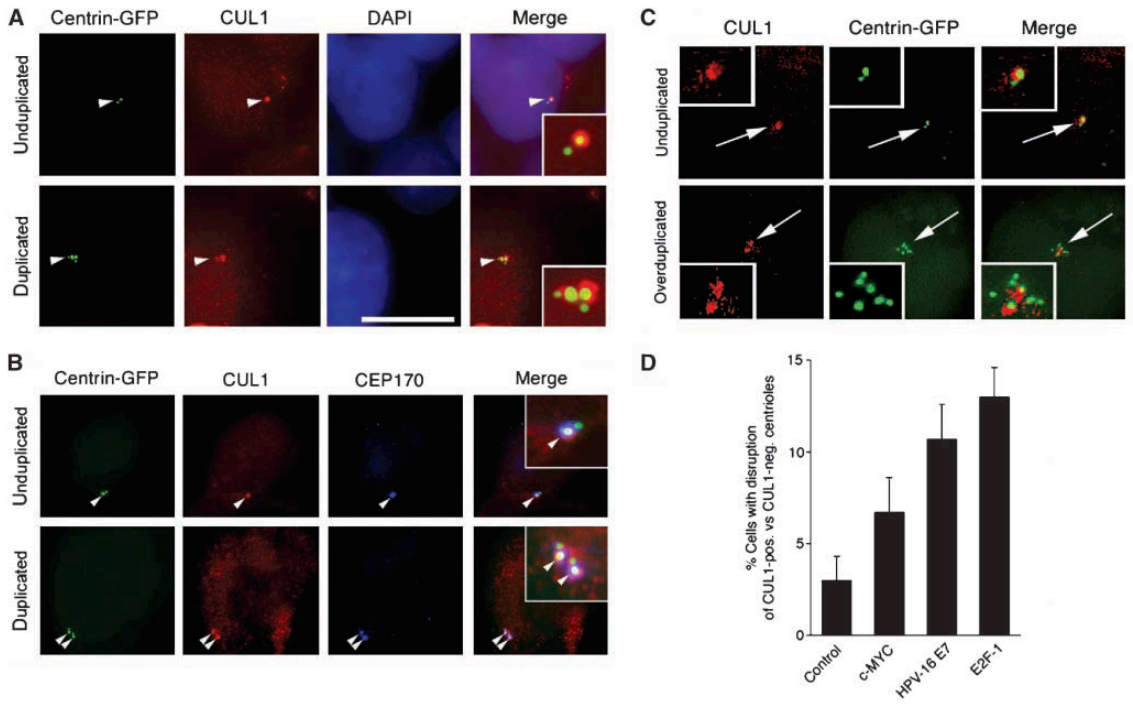

Figure 6. CUL1-positive maternal centrioles serve as platforms for oncogene-induced centriole overduplication.

(A) Immunofluorescence microscopic analysis for CUL1 using U-2 OS cells stably expressing centrin-GFP (bottom panels). Arrows indicate centrioles shown in inserts. Nuclei stained with DAPI. Scale bar indicates 10 μm. (B) Co-immunofluorescence microscopic analysis of U-2 OS/centrin-GFP cells for CUL1 and CEP170, a marker for mature maternal centrioles. Arrowheads indicate centrioles with co-localization of CUL1 and CEP170 (see also inserts). (C) Immunofluorescence microscopic analysis of U-2 OS/centrin-GFP cells for CUL1 following overexpression of E2F-1. Note overduplication of centrioles in the presence of only two CUL1-positive centrioles (bottom panels). (D) Quantification of the proportion of U-2 OS/centrin-GFP cells with centriole overduplication in the presence of one or two CUL1-positive centrioles after overexpression of MYC, HPV-16 E7 or E2F-1. Arrows point to centrioles shown in inserts. Mean and standard error of three independent experiments with at least 100 cells counted per experiment are shown.

These results indicate that the SCF ubiquitin ligase component CUL1 localizes to maternal centrioles that serve as assembly platforms for excessive daughter centriole formation induced by various oncogenes.

Inhibition of SCF ubiquitin ligase activity causes centriole multiplication

To determine the role of CUL1 in the control of centriole biogenesis, we used siRNA to deplete cells of CUL1 and a dominant-negative mutant of CUL1 (DN-CUL1), which has recently been shown to effectively reduce CUL1-based SCF activity [119]. SiRNA-mediated knock-down of CUL1 led to centriole multiplication with an increase of cells showing multiple daughter centrioles at single maternal centrioles (Fig. 7A) from 0.6% in controls to 8.5% in CUL1-depleted cells ($p \leq 0.05$; Fig. 7B, left panel). Centriole multiplication was also detected in cells transiently transfected with DN-CUL1 with an increase from 1.3% in controls to 12% in DN-CUL1-transfected cells ($p \leq 0.05$; Fig. 7B, right panel). CUL1 is involved in the degradation of a number of critical cell cycle regulators including cyclin E [120, 121]. In line with this notion, we detected an increase of cyclin E protein expression in U 2-OS/centrin-GFP cells treated with either siRNA against CUL or DN-CUL1 (Fig. 7C).

These results suggest that cyclin E may contribute to centriole multiplication induced by inhibition of CUL1. However, when cyclin E together with its kinase subunit CDK2 was overexpressed in U-2 OS/centrin-GFP cells, no significant increase of cells with centriole multiplication was detected (Fig. 8A). Only when cyclin E/CDK2 complexes were co-expressed with increasing amounts of the centrosomal kinase PLK4, a significant increase of cells with centriole multiplication was detected (Fig. 8A,B). Co-expression of PLK4 together with cyclin E/CDK2 consistently led to a higher frequency of centriole multiplication than overexpression of PLK4 alone (Fig. 8A,B).

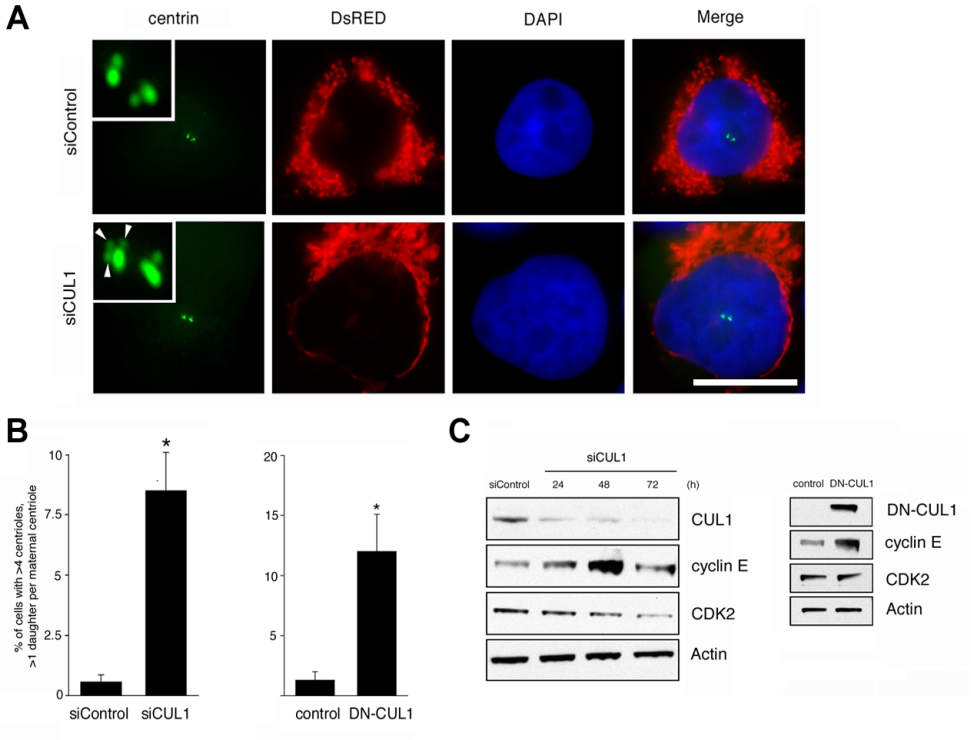

Figure 7. Inhibition of CUL1 causes centriole multiplication.

(A) Fluorescence microscopic analysis of U-2 OS/centrin-GFP cells transfected with either control siRNA duplexes (siControl) or siRNA targeting CUL1 (siCUL1) for 72 h. A DsRED-encoding plasmid was used as transfection marker. Nuclei stained with DAPI. Arrowheads in bottom insert indicate supernumerary daughter centrioles at a single mother. Scale bar indicates 10 μm. (B) Quantification of centriole multiplication (>4 centrioles total, >1 daughter at a single mother) in U-2 OS/centrin-GFP cells transfected with either control (siControl) or CUL1 (siCUL1) siRNA duplexes for 72 h (left panel) or ectopic expression of empty vector control or DN-CUL1 for 48 h (right panel). Mean and standard error of three independent experiments with at least 100 cells counted per experiment are shown. Asterisk indicates statistically significant differences ($p \leq 0.05$; Student's t test for independent samples). (C) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells transiently transfected with siRNA duplexes targeting CUL1 (siCUL1) or control siRNA (siControl) for the indicated time intervals (left panels) or ectopic expression of empty vector control or DN-CUL1 for 48 h (right panel). DN-CUL1 was detected using an OctA-Probe/Flag® antibody (Santa Cruz). Immunoblot for Actin shows protein loading.

Given the known requirements of both cyclin E/CDK2 complexes and PLK4 for efficient centriole overduplication and flower formation [76, 80], we asked whether cyclin E/CDK2 complexes can alter the expression of PLK4 at centrioles. To test this idea, U-2 OS/centrin-GFP cells were transiently transfected with cyclin E and CDK2 followed by immunofluorescence microscopy for endogenous PLK4 protein (Fig. 8C). In empty vector-transfected control cells, disengaged centrioles or duplicated centrioles were associated with a single PLK4 dot that typically localized to the site of daughter centriole synthesis at the wall of the maternal centriole (Fig. 8C - control). In cells ectopically expressing cyclin E/CDK2, an increased proportion of cells with two or more PLK4 dots at single mothers was detected (Fig. 8C - cyclin E/CDK2). These PLK4 dots can precede the actual formation of centrin-containing procentrioles (Fig. 8C, bottom panels), which is in line with previous results suggesting that PLK4 functions early during daughter centriole synthesis [87]. Aberrant PLK4 dots at maternal centrioles were detected in 28 out of 52 cells (53.8%) transfected with cyclin E/CDK2 in comparison to 10 out of 51 cells (19.6%) transfected with empty vector (Fig. 8D).

Taken together, these results suggest that the centriole multiplication induced by inhibition of CUL1 cannot solely be explained by an accumulation of cyclin E but depends also on increased levels of PLK4.

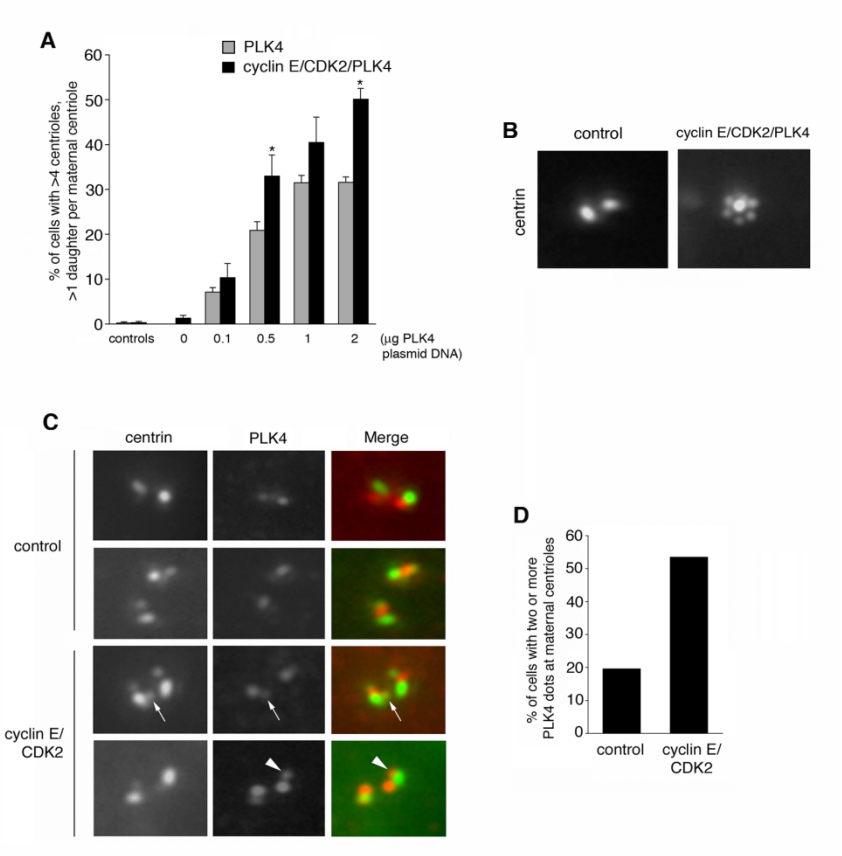

Figure 8. Ectopic expression of cyclin E/CDK2 does not stimulate centriole multiplication but causes aberrant PLK4 recruitment to maternal centrioles.

(A) Quantification of the proportion of cells with centriole multiplication (>4 centrioles, >1 daughter per maternal centriole) after transient transfection with empty vectors (controls) or either cyclin E/CDK2 alone (0 μg PLK4 plasmid DNA) or increasing amounts of PLK4 alone (grey bars) or a combination of cyclin E/CDK2 with increasing amounts of PLK4 plasmid DNA (black bars). Asterisks indicate statistically significant differences ($p \leq 0.05$ at 0.5 μg and $p \leq 0.005$ at 2 μg PLK4 plasmid DNA). (B) Fluorescence microscopic analysis of U-2 OS/centrin-GFP cells after transient transfection with empty vector (control) or cyclin E, CDK2 and PLK4. Note the centriole flower in the right panel.(C) Immunofluorescence microscopic analysis of U-2 OS/centrin-GFP cells for endogenous PLK4 expression after transient transfection with either empty vector (control) or cyclin E/CDK2. Arrows indicate an aberrant daughter centriole that co-localizes with PLK4 at the maternal centriole. Arrowheads indicate an aberrant PLK4 signal at a maternal centriole without a detectable centrin-positive daughter. (D) Quantification of the percentage of cells with aberrant (two or more) PLK4 dots at maternal centriole following transfection with empty vector (control) or cyclin E/CDK2.

PLK4 is degraded by the proteasome and regulated by activation-dependent degradation

Our findings that cyclin E/CDK2 complexes cause an aberrant recruitment of PLK4 to maternal centrioles but do not lead to centriole multiplication unless PLK4 is overexpressed suggest that PLK4 levels are rate-limiting for excessive daughter centriole assembly and hence are tightly regulated in order to suppress aberrant procentriole assembly. To explore how PLK4 protein expression is kept at a low level, we first analyzed U-2 OS/centrin-GFP cells ectopically expressing PLK4 and treated with the proteasome inhibitor Z-L_3VS, which readily stimulates centriole multiplication [80].

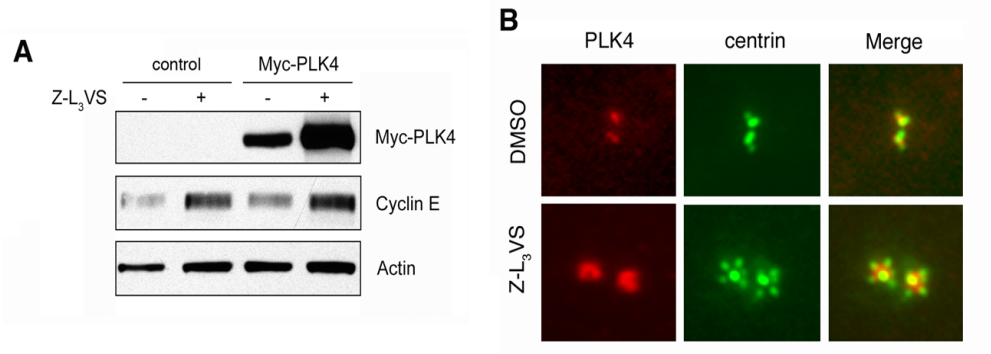

Figure 9. PLK4 is degraded by the proteasome.

(A) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells transiently transfected with either empty vector (control) or PLK4 and treated with 1 μM of the proteasome inhibitor Z-L_3VS or 0.1% DMSO at 24 h after transfection for an additional 24 h. (B) Immunofluorescence microscopic analysis or U-2 OS/centrin-GFP cells treated with 0.1% DMSO or 1 μM Z-L_3VS for 48 h and stained for PLK4. Note the excessive amount of PLK4 at maternal centrioles of centriole flowers.

Immunoblot analyses revealed a stabilization of ectopically expressed PLK4, together with cyclin E, in Z-L_3VS-trated cells (Fig. 9A). In addition, Z-L_3VS treatment led to an excessive amount of endogenous PLK4 at maternal centriole (Fig. 9B). These results indicate that PLK4 protein levels are, at least in part, regulated by the ubiquitin-proteasome machinery.

To further explore why cyclin E/CDK2 can only promote centriole flower formation in the presence of excessive amounts of PLK4, PLK4 protein levels were determined by immunoblotting after ectopic expression of Myc-tagged PLK4 alone or in combination with cyclin E and/or CDK2 (Fig. 10). Surprisingly, we detected a reduction of PLK4 protein levels in the presence of ectopically expressed cyclin E or cyclin E/CDK2 (Fig. 10A). Similar results were obtained in HeLa cells (not shown). In contrast to wild-type PLK4, protein levels of catalytically inactive mutant PLK4 D154A [76] were not significantly decreased in the presence of ectopically expressed cyclin E/CDK2 (Fig. 10A).

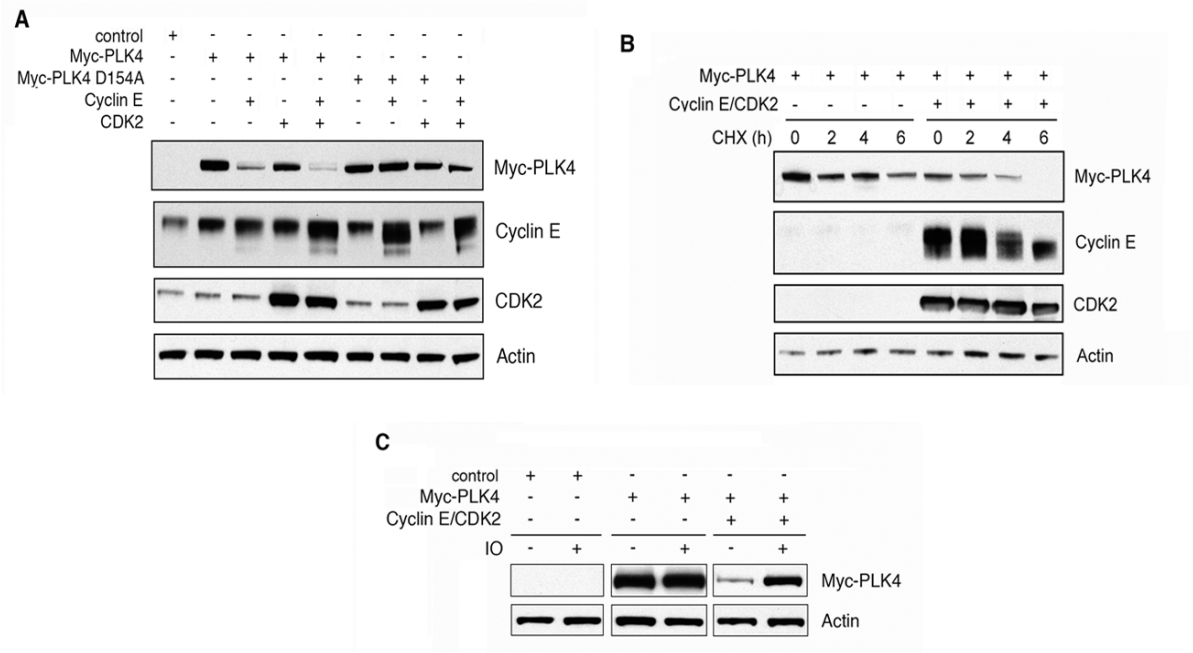

Figure 10. Cyclin E/CDK2 reduce PLK4 protein stability in a PLK4 kinase activity-dependent manner.

(A) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells following transient transfection (48 h) with empty vector (control), Myc-tagged PLK4, Myc-tagged kinase-inactive mutant PLK4 D154A, cyclin E and/or CDK2. Note the decreased protein levels of wild-type PLK4 upon co-transfection with cyclin E or cyclin E/CDK2. No such reduction was detected in cells transfected with catalytically inactive mutant PLK4 D154A. Immunoblots for Actin are shown to demonstrate protein loading (A-C). (B) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells after transient transfection Legend is continued on the following page.

(48 h) with PLK4 alone or PLK4 and cyclin E/CDK2 and treatment with 30 μg/ml cycloheximide (CHX) for the indicated time intervals. Note the decreased PLK4 protein stability in the presence of ectopically expressed cyclin E/CDK2. (C) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells transiently transfected (24 h) with empty vector (control) or PLK4 alone or PLK4 in combination with cyclin E/CDK2 and treatment with 1 μM of the CDK inhibitor indirubin-3'-oxime for 24 h (IO). Note the stabilization of PLK4 protein in IO-treated cells in comparison to controls (0.1% DMSO).

Since PLK4 D154A mutant has no apparent defect in its localization to centrioles [76], we interpret this finding as evidence that the degradation of PLK4 depends on its activation. Similar results have been reported for other protein kinases [122]. The decrease of PLK4 protein levels in the presence of cyclin E/CDK2 was due to decreased protein stability as shown by a cycloheximide block experiment (Fig. 10B). Furthermore, inhibition of CDK activity using the small molecule inhibitor indirubin-3'-oxime (IO) led to a stabilization of PLK4 protein underscoring the CDK dependency of this process (Fig. 8C).

Taken together, these findings suggest cyclin E/CDK2 promote the aberrant recruitment of PLK4 to maternal centrioles followed by its activation-dependent degradation. This process may prevent the accumulation of active PLK4 at maternal centrioles at levels that may promote aberrant procentriole assembly. Our finding that additional PLK4 dots at maternal centrioles are often weaker in signal intensity than normal endogenous PLK4 dots (Fig. 10C) lends support to this notion.

CUL1 regulates PLK4 protein stability

We next asked whether CUL1 contributes to the regulation of PLK4 protein levels. Ectopic expression of DN-CUL1 caused an excess of endogenous PLK4 at maternal centrioles

(Fig. 11A, left panels) that was phenotypically similar to PLK4 accumulation in response to Z-L_3VS-associated inhibition of proteasomal PLK4 degradation. To accurately assess this increase, the integrated density of PLK4 signals at maternal centrioles was quantified using ImageJ and expressed in arbitrary units (http://rsbweb.nih.gov/ij/; Fig. 11A, right panels). Control cells in which a maternal centriole organized the formation of a single daughter (n=32) had a mean integrated signal density of PLK4 of 599 at the mothers. In cells with flower formation after transfection with DN-CUL1 (n=29, average number of daughters = 4.2), the mean integrated PLK4 signal density was significantly increased to 4,114 ($p \leq 0.001$). This value was significantly higher than expected if each daughter was associated with the amount of PLK4 detected in control cells (4.2 x 599 = 2,516) thus underscoring that DN-CUL1 indeed leads to an excessive level of PLK4 protein at maternal centrioles.

We tested next the effects of DN-CUL1 on cyclin E/CDK2-induced PLK4 degradation. Using immunoblotting, we found that DN-CUL1 abrogates the reduction of PLK4 protein levels associated with ectopic expression of cyclin E/CDK2 (Fig. 11B). DN-CUL1 also increased the baseline protein stability of ectopically expressed PLK4 in the absence of overexpressed cyclin E/CDK2 (Fig. 11C). Depletion of CUL1 by siRNA led to similar results (not shown).

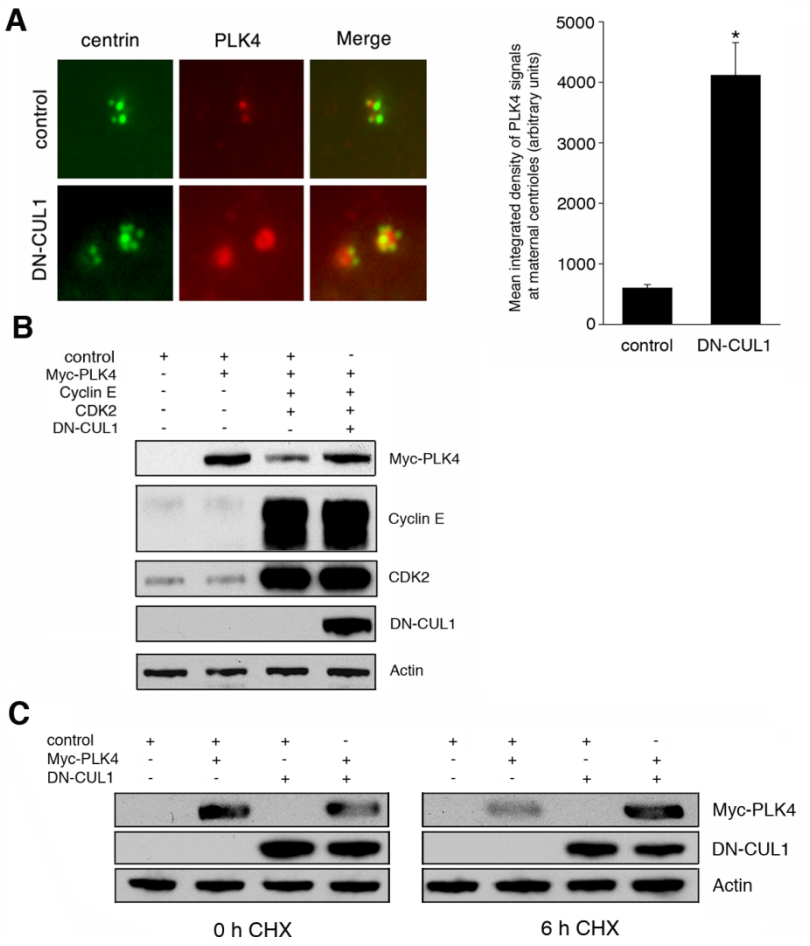

Figure 11. CUL1 regulates PLK4 protein stability.

(A) Immunofluorescence microscopic analysis of U-2 OS/centrin-GFP cells for PLK4 at 48 h after transfection of empty vector (control) or DN-CUL1. Note the excessive amount of PLK4 in the bottom panels. Quantification of the integrated density of PLK4 signals at maternal centrioles in U-2 OS/centrin-GFP cells transfected with empty vector or DN-CUL1 for 48 h. Asterisk indicates statistically significant differences ($p \leq 0.001$; Student's t test for independent samples). (B) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells after transient transfection (48 h) with empty vectors (control) or PLK4 in combination with either cyclin E/CDK2 alone or cyclin E/CDK2 and DN-CUL1. Note the increase of PLK4 protein in the presence of DN-CUL1 in the last lane. (C) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells after transient transfection with empty vectors (control) or dominant-negative CUL1 (DN-CUL1) for 48 h followed by transfection with Myc-PLK4 for 24 h and treatment with 30 μg/ml cycloheximide (CHX) for the indicated time intervals. Note the increased protein stability of Myc-PLK4 in the presence of DN-CUL1 (6 h CHX, last lane).

Taken together, these results suggest that CUL1 is involved in both cyclin E/CDK2-associated degradation of PLK4 and baseline PLK4 protein stability.

CAND1, an inhibitor of SCF-ligase activity, localizes to maternal centrioles and stabilizes PLK4 protein

We have shown that inhibition of SCF complexes leads to centriole multiplication (Fig. 7A) To further explore the importance of SCF activity in maintaining centriole duplication control we examined the role of CAND1, an inhibitor of SCF ligase activity, in restraining centriole biogenesis. We first asked whether CAND1 localizes to the centrosomes utilizing U-2 OS/centrin-GFP cells. We found that CAND1 localizes to centrioles in a throughout the cell division cycle (Fig. 12A). Further, we determined that CAND1 co-localizes with CUL1 in U-2 OS/centrin-GFP cells (Fig. 12B). We next discovered that CAND1, similar to CUL1, localized predominantly to older, mature centrioles as evidenced by co-localization with CEP170 a marker of mature centrioles (Fig. 12C).

Based on these observations, we asked whether ectopic expression of CAND1 would induce centriole multiplication. We found a modest but significant increase in centriole multiplication from 0.5% in controls to 1.5% in CAND1-transfected cells ($p \leq 0.05$) (Fig. 12D). Due to the fact that centriole multiplication was induced by ectopic expression of CAND1, we asked if CAND1 can modulate PLK4 function. We expressed CAND1 in U-2 OS/centrin-GFP cells for 24 h and then transfected cells with increasing amounts of PLK4 for a further 24 h and assayed for centriole multiplication. We found that cells which co-expressed CAND1 and PLK4 together consistently exhibited an enhancement of centriole multiplication over that seen with ectopic expression of PLK4 alone (Fig. 12E, black bars).

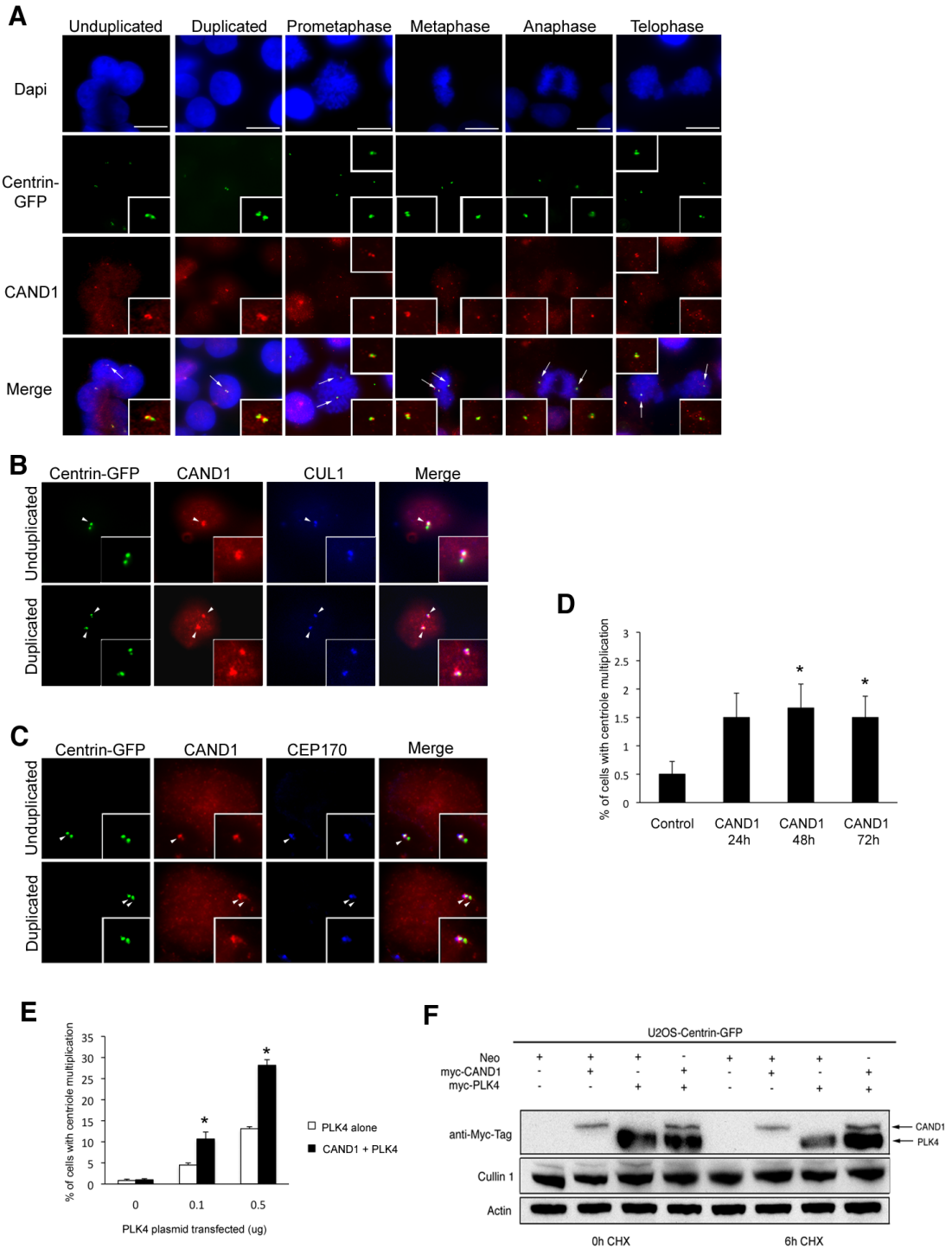

Figure 12. CAND1, an inhibitor of CUL1-based ligase activity, localizes to centrioles and regulates PLK4 stability.

(A) Immunofluorescence microscopic analysis of CAND1 throughout the cell cycle using U-2 OS cells stably expressing centrin-GFP. Arrows indicate centrioles shown in inserts. Legend is continued on the following page.

Nuclei stained with DAPI. Scale bar indicates 10 μm. (B) Co-immunofluorescence microscopic analysis of U-2 OS/centrin-GFP cells for CAND1 and CEP170 a marker of mature maternal centrioles. Arrowheads indicate centrioles with co-localization of CAND1 and CEP170 (see also inserts). (C) Co-immunofluorescence microscopic analysis of U-2 OS/centrin-GFP cells for CAND1 and CUL1. Arrowheads indicate centrioles with co-localization of CAND1 and CUL1 (see also inserts). (D) Quantification of centriole multiplication (>4 centrioles total, >1 daughter at a single mother) in U-2 OS/centrin-GFP cells ectopically expressing either a control plasmid or Myc-CAND1 for 48 h (left panel) Mean and standard error of two independent experiments with at least 100 cells counted per experiment are shown. Asterisk indicates statistically significant differences ($p \leq 0.05$; Student's t test for independent samples). (E) Immunoblot analysis of whole cell extracts from U-2 OS/centrin-GFP cells after transient transfection with empty vectors (control), Myc-CAND1, Myc-PLK4 for 48 h and treatment with 60 μg/ml cycloheximide (CHX) for the indicated time intervals. Note the increased protein stability of Myc-PLK4 in the presence of Myc-CAND1 (6 h CHX, last lane). (F) Quantification of the proportion of cells with centriole multiplication (>4 centrioles, >1 daughter per maternal centriole) after transient transfection with empty vectors (controls) or CAND1 alone (0 μg PLK4 plasmid DNA) or increasing amounts of PLK4 alone (grey bars) or a combination of CAND1 with increasing amounts of PLK4 plasmid DNA (black bars). Asterisks indicate statistically significant differences ($p \leq 0.05$ at 0.1 Õg and $p \leq$ 0.001 at 0.5 μg PLK4 plasmid DNA).

Finally, we asked if CAND1 plays a role in the regulation of PLK4 protein levels. Performing a cycloheximide block experiment, we found that ectopic expression of CAND1 stabilized PLK4 protein levels without affecting CUL1 stabilization (Fig. 12F). Together, these results suggest that CAND1 overexpression modulates PLK4 half-life thereby promoting centriole multiplication.

2.4 DISCUSSION

Results presented in Chapter 2 of this thesis show that the core SCF ubiquitin ligase component CUL1 is critical to tightly regulate PLK4 protein levels in order to suppress aberrant centriole biogenesis at maternal centrioles.

Upregulation of PLK4 expression is an extremely powerful stimulus for centriole multiplication [80, 87, 98, 99]. The fact that overexpression of cyclin E/CDK2 can aberrantly recruit PLK4 to maternal centrioles (Fig. 8C) provides mechanistic insight how CDK2 and PLK4 cooperate to stimulate excessive daughter centriole formation [76, 80]. Our finding that overexpression of cyclin E/CDK2 leads to the degradation of PLK4 in a PLK4 kinase activity-dependent manner explains why cyclin E/CKD2 alone is not sufficient to trigger centriole multiplication. The low endogenous levels of PLK4 together with the activation-dependent degradation of PLK4 hence likely represent cellular failsafe mechanisms to limit normal centriole biogenesis to one per cell division cycle.

We were not able to demonstrate a direct interaction between CDK2 and PLK4 by co-immunoprecipitation (data not shown) suggesting that the association is either transient or indirect. Whether any of the reported centrosomal CDK2 targets such as nucleophosmin, CP110 or MPS1 play a role in this process remains to be determined [123-126].

The question whether normal activation of cyclin E/CDK2 complexes triggers PLK4 recruitment during undisturbed, normal centriole duplication also remains to be determined. It is interesting to note, however, that CDK2 deficient MEFs cells have no detectable defects in centrosomal duplication [127]. Oncogene-triggered centrosome overduplication, however, was clearly blocked in CDK2-deficient cells [127] suggesting that deregulation of CDK2, as frequently seen in tumors, may promote centriole overduplication by aberrantly recruiting PLK4.

One prediction from our results is that such tumor cells may also harbor alterations of the PLK4 degradation machinery. Moreover, oncogenic stimuli that induce centriole overduplication may not only function by deregulation cyclin E/CDK2 activity but also by impacting on CUL1-based SCF ubiquitin ligase activity (Fig. 11D).

A possible mechanism for such inhibition may be the CUL1 neddylation cycle or the association/dissociation with the CUL1 inhibitor CAND1 (Fig. 12C) [112]. Association with CAND1, unlike the neddylation cycle, modulates SCF-mediated proteolytic activity through enhancing the rate of SCF-complex turnover. Paradoxically, CAND1 has been shown to both *in vitro* inhibit and *in vivo* activate SCF complexes [94]. Two mechanisms have been proposed to reconcile these observations. The CUL1 backbone is the rate-limiting factor in SCF complex assembly, as there are over 76 different human F-box proteins currently classified [128]. F-box proteins must complete for binding to the stable CUL1 core and are known to auto-ubiquitinate, promoting their own degradation [94, 129, 130]. It has been proposed that CAND1 association with CUL1 may protect F-box proteins from auto-ubiquitination promoting the efficient assembly of SCF complexes [96]. The second proposal hypothesizes that CAND1 may actually alter the composition of SCF-ligase complexes enhancing the incorporation of particular F-box proteins over others [113]. Our results have shown that overexpression of CAND1 induces a small, but significant, amount of centriole multiplication and modulates PLK4 protein stability (Fig 13E, F). Determining the F-box responsible for PLK4 degradation in human cells will provide mechanistic insight into exactly how CAND1 may regulate SCF activity at maternal centrioles.

Two recent studies in Drosophila cells have demonstrated a role of the SCF/Slimb ubiquitin ligase in the regulation of SAK (PLK4) [99, 131]. Results shown here extend these

findings by substantiating the involvement of SCF ubiquitin ligase complexes in PLK4 regulation in human cells, showing that PLK4 functions as a nexus between the cell cycle engine and the ubiquitin/proteasome machinery. Slimb is the ortholog of the mammalian F-box protein β-TrCP and although knock-out of β-TrCP in mice has been found to lead to centrosome amplification [132], our own experiments did not show significant centriole multiplication after siRNA-mediated knock-down of β-TrCP in human U-2 OS cells (not shown). This finding does not preclude a role of $SCF^{\beta\text{-}TrCP}$ in PLK4 regulation in human cells but it raises the interesting possibility that there may be cell type specific differences in PLK4 regulation and/or that more than one F-box protein participates in SCF-mediated regulation of PLK4 stability in a spatio-temporally controlled manner. Further experiments to explore this possibility in activation-dependent versus baseline PLK4 degradation by CUL1 shown here are clearly warranted. Given the need to tightly regulate PLK4 levels, it is likely that multiple mechanisms converge on PLK4 expression control and protein stability.

The results described here have major implications for the potential mechanism by which the HPV-16 E7 oncoprotein induces centriole multiplication. The high-risk HPV-16 E7 protein is known to deregulate cyclin E/CDK2 complexes and has been show to localize to the centrosome [50, 82, 133]. Whether HPV-16 E7 also deregulates PLK4 protein expression or interferes with SCF-mediated proteolytic control at maternal centrioles is the subject of the following chapter.

3.0 THE HPV-16 E7 ONCOPROTEIN INDUCES CENTRIOLE MULTIPLICATION THROUGH DISRUPTION OF PLK4

3.1 INTRODUCTION

As detailed in Chapter 1 of this thesis, expression of the high-risk HPV-16 E7 oncoprotein promotes genomic instability through the rapid disruption of centriole duplication control. This rapid induction of centriole overduplication occurs through the simultaneous formation of multiple daughter centrioles at single maternal centrioles (centriole multiplication). The exact cellular components that HPV-16 E7 targets to promote centriole multiplication are currently unknown.

Results described in Chapter 2 of this thesis, revealed that several molecular players involved in the centriole multiplication pathway were recently determined. We discovered that CDK2, cyclin E, and PLK4 were necessary factors for centriole multiplication [80]. Ectopic expression of CDK2/cyclin E alone, despite its ability to mediate the aberrant recruitment of PLK4, was not sufficient to induce centriole multiplication. This only occurred when PLK4 was upregulated, suggesting that PLK4 protein levels are rate-limiting for centriole multiplication [134].

High-risk HPV-16 E7 protein expression is known to deregulate cyclin E/CDK2 complexes [50, 133]. Whether HPV-16 E7 also deregulates PLK4 protein expression had not

been determined. However, previous studies have shown that an HPV-16 E7 mutant deficient in pRB binding and degradation is unable to promote centriole overduplication [81]. This observation suggested that disruption of pRB signaling may be important in the ability of high-risk HPV-16 E7 to either target or disrupt the molecular players involved in activation of the centriole multiplication pathway.

PLK4 is an essential regulator of both centriole duplication and cell viability [76, 135]. Deregulation of PLK4 by overexpression, depletion via epigenetic silencing or loss of heterozygosity (LOH) has been associated with chromosomal instability and malignancy [76, 136]. These observations suggest that a strict control of PLK4 transcript and protein levels is necessary to maintain cell viability and that changes in PLK4 regulation are detrimental to genomic integrity.

We report here that the PLK4 protein is aberrantly recruited to maternal centrioles in primary human keratinocytes engineered to stably express HPV-16 E7. Real-time quantitative reverse transcriptase (qRT-PCR) demonstrated an increase in PLK4 transcription in keratinocytes stably expressing HPV-16 E7. A mutational analysis revealed that the ability of HPV-16 E7 to upregulate PLK4 mRNA was found to be dependent on its ability to degrade pRB and inactivate HDACs, suggesting a role for E2F-mediated gene transcription in deregulation of PLK4. Collectively, these results highlight the critical role of PLK4 as a regulator of centriole biogenesis and identify PLK4 as a novel target for small molecules to prevent centriole abnormalities, mitotic infidelity and malignant progression in HPV-associated neoplasms.

3.2 MATERIALS AND METHODS

Cell culture, transfections and inhibitor treatments

U-2 OS/centrin-GFP cells (centrin-GFP plasmid was kindly provided by Michel Bornens, Institut Curie, France [114]) were maintained as previously reported [80]. Normal immortalized human keratinocytes (NIKs) engineered to stably express a control plasmid, LXSN, or HPV-16 E7 (generous gift of Susanne Wells, Cincinnati Children's Hospital Medical Center) were maintained in serum-free keratinocyte growth medium (Epilife; Cascade Biologics/Invitrogen) supplemented with human keratinocyte growth supplement (Invitrogen), 50 units/mL penicillin (Cambrex), 50 μ g/mL streptomycin (Cambrex), and fungizone (Invitrogen). For transient transfection of U-2 OS/centrin-GFP cells (48 h), pCMV-based plasmids encoding HPV-16 E7, HPV-16 E7 Δ21-24, HPV-16 E7 L67R (kindly provided by Karl Münger, The Channing Laboratory, Brigham and Women's Hospital, Boston, MA), HPV-6 E6, HPV-6 E7 (LXSN-based low risk HPV-6 E6 and HPV-6 E7 constructs were kindly provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) and sucloned into pCMV-based vectors [137]) or empty vector controls were used and transfected by lipofection (Fugene 6; Roche). Cells were co-transfected with a vector encoding red fluorescent protein targeted to mitochondria (DsRED; BD Biosciences Clontech, Palo Alto, CA) as a transfection marker. For transient transfection (48h) of NIK cells, centrin-GFP was used and transfected utilizing nucleofection (Amaxa).

Immunological Methods

Immunofluorescence staining using PLK4 (mouse monoclonal antibody kindly provided by Erich A. Nigg, Max Planck Institute of Biochemistry, Martinsried, Germany) or centrin

(mouse monoclonal antibody kindly provided by Jeffrey L. Salisbury, Mayo Clinic, Rochester, MN) antibody was performed as described previously [80]. Primary antibody was detected with either Rhodamine Red- or FITC-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) as previously described [80].

Quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR)

RNA was extracted using the RNase Easy kit (Qiagen, USA). DNase I-treated total RNA (500 ng) was subjected to qRT-PCR analysis using the one step QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, USA) and the Real-Time thermocycler iQ5 (BioRad, USA). For qRT-PCR analysis of *PLK4* mRNA, the following primer was used: forward 5′-AGTGCTCCCTTTTTCCCAAT-3′ and reverse 5′-AGCAGCACTATGCATGACCA-3′ (147-bp product). The primer sequences for the control housekeeping β-actin used was: forward 5′-TGCGCAGAAAACAAGATGAG-3′ and reverse 5′-CACCTTCACCGTTCCAGTTT-3′ (113-bp product). All reactions were done in triplicate and relative expression of RNAs was calculated using the ΔΔCT method [138].

Statistical Methods

Student's t test for independent samples was used to calculate statistical significance of centriole counts. Chi-square test was used to determine the statistical significance of aberrant PLK4 recruitment to maternal centrioles in NIK cells.

3.3 RESULTS

PLK4 is aberrantly recruited to maternal centrioles in HPV-16 E7 expression cells

It was recently discovered that deregulation of cyclin E/CDK2 led to the aberrant recruitment of PLK4 to maternal centrioles and that PLK4 protein level is the rate-limiting step in the centriole multiplication pathway [134]. We therefore examined if HPV-16 E7 expression resulted in the aberrant recruitment of PLK4 protein to maternal centrioles. Non-transformed normal immortalized human keratinocytes (NIKs) engineered to stably express either a control plasmid, LXSN, or HPV-16 E7 were transiently transfected with a centrin-GFP plasmid as a marker of centrioles followed by immunofluorescence microscopy for endogenous PLK4. In control LXSN-expressing cells, disengaged centrioles were associated with a single dot of endogenous PLK4 that localized to the site of daughter centriole synthesis at the wall of maternal centrioles (Fig. 13A, NIK LXSN). In NIKs stably expressing HPV-16 E7 an increased proportion of cells with two or more dots of endogenous PLK4 at single mothers was detected (Fig. 13A, NIK HPV-16 E7). These aberrant PLK4 dots were occasionally found to precede the formation of daughter centrioles, as previously noted [134]. Aberrant PLK4 dots at maternal centrioles were detected in 29 of 86 (33.7%) cells expressing the control plasmid LXSN (Fig. 13B). Stable expression of HPV-16 E7 led to aberrant PLK4 expression at 45 of 89 (50.6%) of NIKs ($p \leq 0.05$).

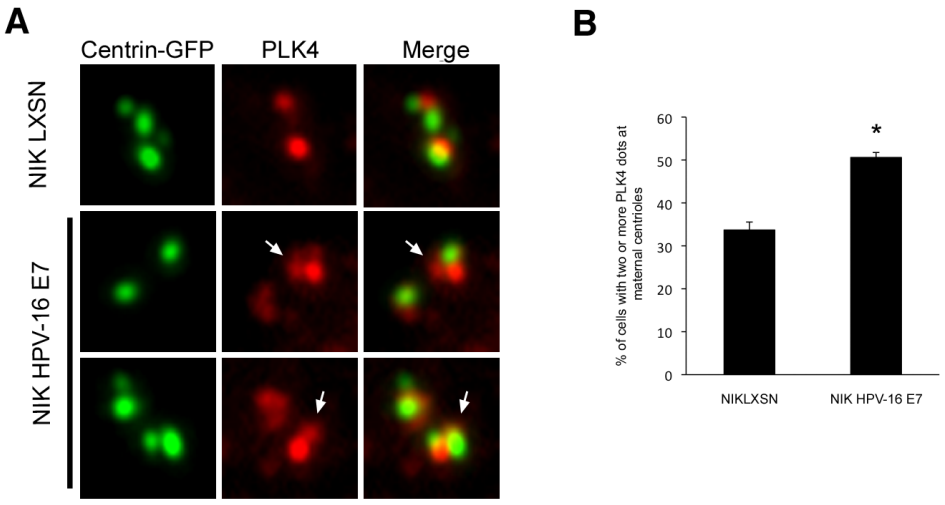

Figure 13. Stable expression of HPV-16 E7 leads to the aberrant recruitment of PLK4 to maternal centrioles. (A) Immunofluorescence microscopic analysis of NIKs transiently transfected with centrin-GFP (48 h) for endogenous PLK4 expression. Arrows indicate an aberrant PLK4 signal at a maternal centriole. (B) Quantification of the percentage of cells with aberrant (two or more) PLK4 dots at maternal centrioles in NIKs stably expressing either a control plasmid, LXSN, or HPV-16 E7. Asterisks indicate statistically significant differences ($p \leq 0.05$).

These results suggest that HPV-16 E7 expression leads to the aberrant recruitment of PLK4 to maternal centrioles.

HPV-16 E7 expression upregulates PLK4 mRNA levels

Previous results have shown that aberrant recruitment of endogenous PLK4 protein was insufficient to induce centriole multiplication. A concomitant increase in PLK4 protein level was needed for centriole multiplication to occur [134]. The aberrant recruitment of PLK4 to maternal centrioles is recapitulated in cells stably expressing HPV-16 E7 (Fig. 13A). We next asked if these cells also exhibited centriole overduplication. Stable expression of HPV-16 E7 in NIKs led to a statistically significant increase in centriole overduplication from 3.6% in control LXSN expressing cells to 8.3% in HPV-16 E7 expressing cells ($p \leq 0.05$) (Fig. 14A-B). This result

suggests that the aberrant recruitment of PLK4 protein to maternal centrioles in cells stably expressing HPV-16 E7 may promote centriole overduplication and that PLK4 protein levels may be increased in these cells.

We next wanted to determine if NIKs stably expressing HPV-16 E7 deregulated PLK4 expression to ultimately enhance PLK4 protein levels at maternal centrioles. One of the most important functions of the HPV E7 oncoprotein is to disrupt cellular gene transcription. The PLK4 promoter is known to contain E2F-responsive elements leading to the hypothesis that HPV-16 E7 expression may increase PLK4 gene transcription [139]. To test this, we performed real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of PLK4 mRNA in NIKs stably expressing either a control plasmid, LXSN, or HPV-16 E7. We found that stable expression of HPV-16 E7 led to a small (1.25-fold) increase in PLK4 mRNA over control LXSN-expressing cells (Fig. 14C).

These results indicate that stable expression of HPV-16 E7 in non-transformed cell leads to upregulation of PLK4 mRNA which may promote PLK4 protein accumulation at maternal centrioles.

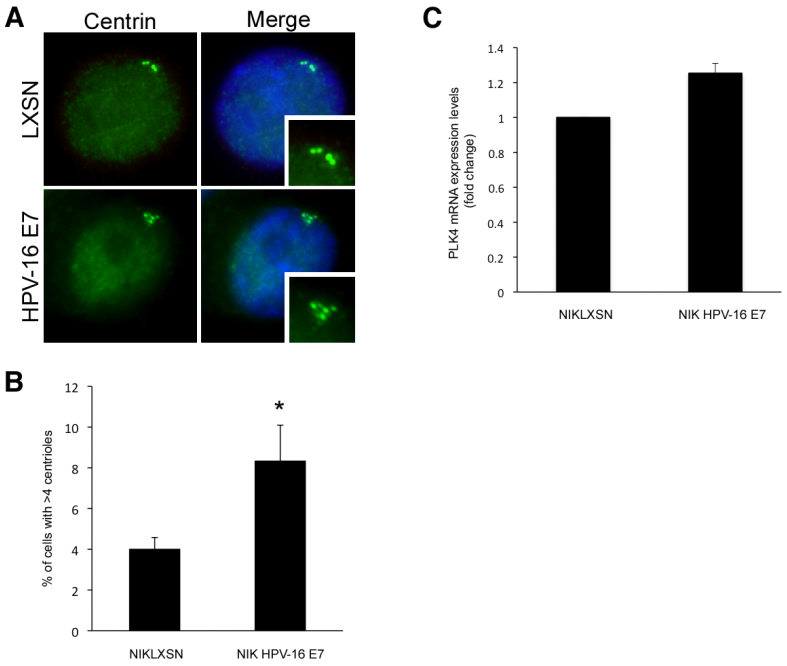

Figure 14. NIKs stably expressing HPV-16 E7 exhibit centriole overduplication and upregulate PLK4 mRNA. (A) Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for PLK4 was performed on total RNA isolated from NIKs expressing either a control plasmid, LXSN, or HPV-16 E7. β–actin was used as a control housekeeping gene. Experiments were performed in triplicate and analyzed as described in the materials and methods section. (B) Immunofluorescence and quantification of NIKs stably expressing either a control plasmid, LXSN, or HPV-16 E7 exhibiting >4 centrioles. Centrioles in NIKs were visualized by staining with centrin antibody. Asterisks indicate statistically significant differences ($p \leq 0.05$).

Upregulation of PLK4 mRNA by HPV-16 E7 is pRB- and HDAC-dependent

We next explored which functional domain of HPV-16 E7 was responsible for upregulation of PLK4 mRNA. We transiently transfected U-2 OS/centrin-GFP cells with various HPV-16 E7 mutant constructs and isolated total RNA to perform qRT-PCR of PLK4 mRNA. Similar to stable expression in NIKs, transient expression of HPV-16 E7 led to a small (1.7 fold) increase in PLK4 mRNA (Fig. 15A). An HPV-16 E7 mutant with deletion of the amino acid region 21-24 which contains the LCXCE motif (HPV-16 E7 Δ21-24) and is deficient in pRB

binding was unable to upregulate PLK4 mRNA [42, 140] (Fig. 15A). Further, an HPV-16 E7 amino acid substitution mutant L67R (HPV-16 E7 L67R) which is incapable of interaction with histone deacetylases (HDACs) was also deficient in modulating PLK4 mRNA level (Fig. 15A).

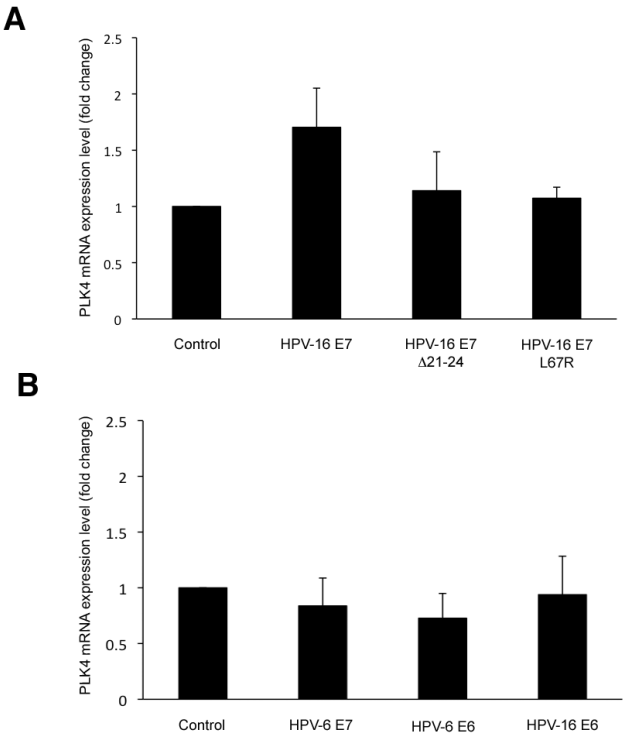

Figure 15. The ability of HPV-16 E7 to upregulate PLK4 mRNA is both pRB- and HDAC-dependent.

(A) Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for PLK4 was performed on total RNA isolated from U 2-OS/centrin-GFP cells transiently expressing either an empty vector control (Control), HPV-16 E7, mutant HPV-16 Δ21-24 or mutant HPV-16 E7 L67R. β-actin was used as a control housekeeping gene. Experiments were performed in triplicate and analyzed as described in the materials and methods section. (B) Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for PLK4 was performed on total RNA isolated U 2-OS/centrin-GFP cells transiently expressing either an empty vector control (Control), HPV-6 E7, HPV-6 E6, or HPV-16 E6. β-actin was used as a control housekeeping gene. Experiments were performed in triplicate and analyzed as described in the materials and methods section.

Importantly, PLK4 mRNA level was not increased following transient expression of low-risk HPV-6 E7, high-risk HPV-16 E6 or low-risk HPV-6 E6 (Fig. 15B).

Together, our results suggest that the induction of centriole multiplication by the HPV-16 E7 oncoprotein relies, at least in part, on the upregulation of PLK4 mRNA in both a pRB- and HDAC-dependent manner.

The ability of HPV-16 E7 to induce centriole overduplication correlates with upregulation of PLK4 mRNA

We next analyzed whether the ability of the HPV-16 E7 oncoprotein to upregulate PLK4 mRNA correlated with the capacity to induce centriole overduplication. Transient expression in U-2 OS/centrin GFP cells led to an increase in centriole overduplication from 1% in control transfected cells to 6.5% in HPV-16 E7 transfected cells ($p \leq 0.001$; Fig 16A-B). Expression of HPV-16 E7 Δ21-24 completely abolished the ability of the E7 oncoprotein to induce centriole overduplication, as has been previously reported (Fig. 16); [72, 81]). Interestingly, expression of HPV-16 E7 L67R led to a 1.3-fold (2.3%) increase in cells exhibiting centriole overduplication compared to control transfected cells (Fig. 16B). In conclusion, upregulation of PLK4 mRNA correlates with the ability of HPV-16 E7 to promote centriole overduplication.

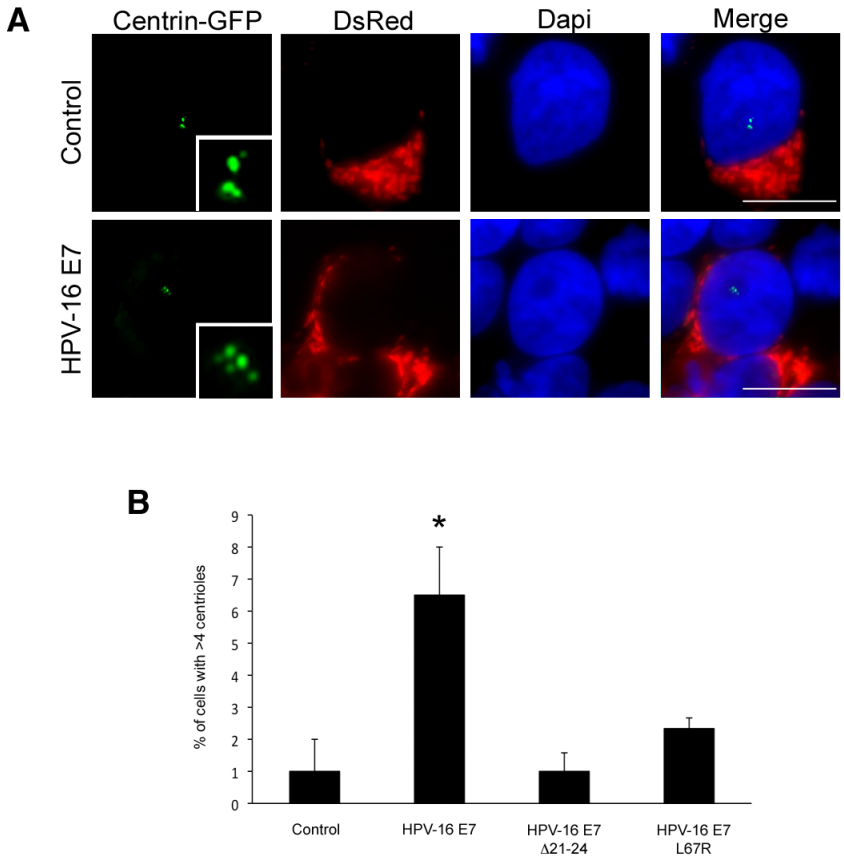

Figure 16. Enhancement of PLK4 mRNA by HPV-16 E7 mutant constructs correlates with their ability to induce centriole overduplication.

(A) Fluorescence microscopic analysis of U-2 OS/centrin-GFP cells transfected with either an empty vector control plasmid (Control) or HPV-16 E7 for 48 h. A DsRED-encoding plasmid was used as transfection marker. Nuclei stained with DAPI. Scale bar indicates 10 μm. (B) Quantification of centriole overduplication (>4 centrioles) in U-2 OS/centrin-GFP cells ectopically expressing either a control plasmid, HPV-16 E7, mutant HPV-16 Δ21-24 or mutant HPV-16 E7 L67R for 48 h. Mean and standard error of two independent experiments with at least 100 cells counted per experiment are shown. Asterisk indicates statistically significant differences ($p \leq 0.001$; Student's t test for independent samples).

3.4 DISCUSSION

Results described here suggest a mechanism for the rapid induction of centriole multiplication by the HPV-16 E7 oncoprotein. It has previously been shown that cyclin E/CDK2 mediated the aberrant recruitment of PLK4 to maternal centrioles. However a concurrent increase in PLK4 protein was necessary for centriole multiplication to occur suggesting that the early steps of daughter centriole synthesis are reversible. We discovered that the aberrant recruitment of PLK4 to maternal centrioles is recapitulated in cells stably expressing HPV-16 E7 and that these cells contained increased PLK4 mRNA transcripts.

The increase in PLK4 mRNA, albeit only modest, may be necessary to promote the aberrant recruitment of excess PLK4 to maternal centrioles in the form of multiple PLK4 dots and ultimately centriole multiplication. Support for this notion comes from a previous study which determined that ongoing RNA polymerase II transcription is necessary for HPV-16 E7 induced centriole overduplication but dispensable for normal centriole duplication [141]. Although we do not know if PLK4 transcription is RNA polymerase II dependent, this is in line with our finding that increased PLK4 mRNA transcripts play a role in HPV-16 E7-induced centriole multiplication. However, further experiments utilizing siRNA against PLK4 in HPV-16 E7 expressing cells are necessary to confirm this hypothesis.

The PLK4 promoter has been shown to contain E2F-responsive elements and be repressed by HDACs [142]. In line with this, we found that the HPV-16 E7 Δ21-24 mutant which is incapable of pRB-binding was unable to upregulate PLK4 mRNA and, in accordance with previous studies, unable to promote centriole overduplication [81]. Although the HPV-16 E7 Δ21-24 mutant, is unable to bind and degrade pRB, it is still capable of interacting with HDACs [42]. However, this interaction may be irrelevant in this context as derepression of E2F-

gene promoters would not automatically increase their transcriptional expression without a concurrent disruption of the pRB signaling axis.

Like the HPV-16 E7 Δ21-24 mutant construct, the HDAC deficient mutant L67R was also unable to upregulate PLK4 mRNA. The HPV-16 L67R mutant is still capable of interacting with pRB although it does so less efficiently and has a reduced capacity to activate E2F-dependent transcription [44, 140]. This defect complicates analysis of the role of HDACs in the HPV-16 E7-induced modulation of PLK4 mRNA levels. Analyzing PLK4 mRNA abundance in HPV-16 E7 expressing cell which are deficient in HDACs or pRB-family members would clarify the role of HDACs in the HPV-16 E7–mediated modulation of PLK4 mRNA.

However, unlike the pRB-binding deficient mutant, the HPV-16 L67R mutant was still able to induce a small but significant increase in centriole overduplication. This may be explained by the fact that the pRB-binding region within Δ21-24 has been implicated in HPV-16 E7 mediated pRB-independent promotion of centriole overduplication [82]. The small amount of centriole overduplication seen following HPV-16 E7 L67R may be due to this pRB-independent mechanism. There are several reports suggesting that mutations in the zinc-finger domain of HPV-16 E7 result in reduced protein stability [43, 140]. Decreased protein stability of HPV-16 L67R compared to wild-type HPV-16 E7 may be a further reason why this mutant construct did not induce a more significant amount of centriole overduplication.

An alternative, although not mutually exclusive, mechanism of HPV-16 E7-mediated increase in PLK4 protein levels is modulation of the proteolytic control system localized at maternal centrioles responsible for regulating PLK4. Experiments described in Chapter 2 of this thesis demonstrated that CUL1, the core component of SCF ubiquitin ligase complexes, localizes to the maternal centriole and regulates PLK4 protein levels to restrain centriole biogenesis [134].

This leads to the question of why excess PLK4 protein recruited to maternal centrioles in HPV-16 E7 expressing cells is not degraded by this proteolytic system to prevent centriole multiplication. HPV-16 E7 has been shown to localize to the centrosome and may locally influence the activity of SCF complexes at maternal centrioles. There is one report, which has been challenged, suggesting that the HPV-16 E7 oncoprotein may directly interact with CUL1 [35, 143]. The CUL1 inhibitor CAND1 has also been implicated in regulation of PLK4 stability (see Chapter 2). Whether CAND1 protein levels are increased in HPV-16 E7 expressing cells, possibly dampening the proteolytic response to excess PLK4 protein recruitment and promoting the accumulation of PLK4 protein levels, remains to be determined.

PLK4 is essential in order to maintain both centriole duplication control and cell viability. Interestingly, the PLK4 locus has not been definitively implicated in a human malignancy [136]. This may be due to the fact that any large scale change in PLK4 level, be it overexpression or insufficiency, is lethal to the cell [136]. This emphasizes that only small changes in PLK4 protein level would be tolerable to the cell and could possibly promote a sub-lethal level of genomic instability setting the stage for malignant progression [76, 136].

We have previously shown that overexpression of minute amounts of PLK4 still leads to a significant increase in centriole multiplication (Chapter 2; [134]. This illustrates that slight changes in PLK4 protein levels, as may occur following the moderate increase in PLK4 mRNA level induced by HPV-16 E7 expression or through potential dampening of the proteolytic response at maternal centrioles, may constitute a large enough change to promote centriole multiplication in HPV-16 E7 expressing cells.

Collectively, these results highlight the critical role of PLK4 as a regulator of centriole biogenesis and identify PLK4 as a novel target for small molecules to prevent centriole abnormalities, mitotic infidelity and malignant progression in pre-invasive HPV-associated neoplasms.

4.0 DAUGHTER CENTRIOLE ELONGATION IS CONTOLLED BY PROTEOLYSIS

A revised manuscript of the work described in this section has been submitted to *Molecular Biology of the Cell* with authors Nina Korzeniewski, Anette Duensing, and Stefan Duensing

N. Korzeniewski performed all experiments described in this section. A. Duensing helped with the experimental design and data analysis. N. Korzeniewski and S. Duensing conceived the project, analyzed the results and wrote the manuscript.

4.1 INTRODUCTION

While we were examining HPV-16 E7 expressing cells for centriole duplication defects we observed a small percentage of cells (~1%) with elongated centrioles (data not shown) which we never saw in control cells. This was intriguing because the HPV-16 E7 oncoprotein is known to manipulate CUL2-containing ubiquitin ligase complexes, replacing the VHL adaptor protein to direct the atypical degradation of pRB [35]. VHL-containing E3 ubiquitin ligases have been implicated in cilia maintenance [144]. We hypothesized that modulation of VHL-containing complexes by HPV-16 E7 expression might induce a small number of cycling cells to aberrantly form primary cilia. The rational for this hypothesis was further strengthened by the recent

discovery that depletion of two centriolar proteins, Cep97 and CP110, led to the inappropriate formation of primary cilia in non-quiescent cells [145]. In many vertebrate cells that have exited the cell cycle and are not actively proliferating, the centriole pair will migrate to the cell surface where the maternal centriole forms a basal body that organizes the elongation and formation of a primary cilium [146]. The primary cilium plays an important role as a cellular sensor providing a signaling platform that modulates both cell cycle progression and cellular differentiation [146]. Cilia dysfunction is associated with several human diseases classified as "ciliopathies" and has also been linked to the development of cancer [147].

Determining what proteins are involved in centriolar elongation, a key step in cilia formation, may aid in determining how and why cilia dysfunctions arise possibly contributing to their therapeutic prevention.

Besides recent studies implicating the centriolar protein CPAP, and the previously identified cilia-suppressive functions of Cep97 and CP110, little is currently about how centriole length is controlled [145, 148-150]. Daughter centriole elongation begins during S-phase of the cell cycle and centrioles reach approximately 80% the length of the maternal centriole in late-G_2 [151]. The daughter centriole reaches the full length of the maternal centriole, but normally not beyond, during the following cell cycle [73]. The mechanism(s) preventing the daughter centriole from elongating to a longer length than the maternal centriole are unknown.

We found that treatment of U-2 OS/centrin-GFP cells with the proteasome inhibitor Z-L_3VS induced abnormally elongated centrioles. This observation, coupled with the link between HPV-16 E7 and modulation of VHL containing E3 ubiquitin ligases activity, we decided to utilize a panel of proteasome inhibitors to examine the role of proteolysis in the control of centriole elongation and possibly cilia formation. We observed that cells exposed to either Z-

L_3VS or MG132 contain abnormally elongated daughter centrioles. Unexpectedly, we found that the elongated centrioles were daughter centrioles, and not elongated mother centrioles as occurs in the case of primary cilia. Abnormally elongated daughter centrioles were found to reach approximately four times the length of normal daughter centrioles. We demonstrate that the ability of Z-L_3VS and MG132 to potently induce daughter centriole elongation correlates with sustained inhibition of the 26S proteasome. Other proteasome inhibitors were also able to induce abnormal centriole elongation, although to lesser extents. The fact that specific and potent proteasome inhibitors were less potent effectors of abnormal centriole elongation suggests a role of non-proteasomal activities, such as those against cellular proteases, in centriole length control. Combining our assay system of Z-L_3VS-induced centriole elongation with an siRNA screen targeting 127 known centrosomal proteins [152], we were able to identify seven centrosomal proteins that attenuated daughter centriole elongation when knocked-down (FOP, CAP350, CPAP, hSAS-6, Cep170, ninein, and C-Nap1) and two centrosomal proteins that promoted this process when depleted (Cep97 and CP110). Our results reveal an unexpected complexity of daughter centriole length control and highlight the critical role of proteolysis in this process.

4.2 MATERIALS AND METHODS

Antibodies

Rabbit anti-Cep97 and CP110 were a kind gift from Brian Dynlacht (NYU Cancer Institute, New York, NY, USA) [145]. Mouse anti-Cep170 and mouse anti-ninein were a kind gift from Erich A. Nigg (Max Plank Institute of Biochemistry, Martinsried, Germany) [77]. Rabbit anti-CPAP was a kind gift from Tang K. Tang (Institute of Biomedical Sciences, Taipei,

Taiwan) [153]. Rabbit anti-CAP350 was obtained commercially from Novus Biologicals (Littleton, CO, USA). Rabbit anti-FGFR1OP was obtained commercially from Proteintech Group (Chicago, IL, USA). Mouse anti-C-Nap1 was obtained commercially from BD Transduction Laboratories (San Jose, CA, USA) and mouse anti-hSAS6 was obtained commercially from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and inhibitor treatment

The human U-2 OS osteosarcoma cells line was obtained from ATCC and maintained in Dulbecco's modified Eagle medium (DMEM; Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA), 50 units/ml penicillin, and 50 μg/ml streptomycin (Cambrex). U-2 OS cells were engineered to stably express a centrin-GFP-encoding construct (kindly provided by Michel Bornens, Institut Curie, Paris, France). Proteasome inhibitors were used at the following concentrations that were associated with at least 50% viable cells after 72 h: Z-L_3VS (used at a 1 μM concentration; Biomol, Plymouth Meeting, PA, USA), MG132 (used at a 1 μM concentration), MG262 (used at a 0.001 μM concentration), lactacystin (used at a 1 μM concentration; all Boston Biochem, Cambridge, MA, USA) and epoxomicin (used at a 0.01 μM concentration; Calbiochem, San Diego, CA, USA) were dissolved in DMSO. In all experiments, solvent controls were included using 0.1% DMSO.

Small interfering RNA (siRNA)

Proteins to be tested in the siRNA screen were depleted using RNA duplexes (Qiagen, Valencia, CA, USA) targeting known centriolar associated proteins as described previously [152]. For the centriole elongation screen, U-2 OS/centrin-GFP cells were grown in

12-well tissue culture plates, on coverslips, with 0.5 mL DMEM free of antibiotics. Cells were transfected with 3 μL of 20 μM annealed RNA duplexes using Oligofectamine (Invitrogen, Carlsbad, CA, USA) transfection reagent. 24 h post transfection cells were treated with 1 μM of the proteasome inhibitor Z-L_3VS and were analyzed 72 h post inhibitor addition. Primary and secondary siRNA screens were performed using target sequences shown in Suppl. Table 1. RNA duplexes that yielded a more than 20% increase or decrease of the proportion of cells with elongated centrioles normalized to Z-L_3VS-treated, control siRNA-transfected cells in both rounds of screens were considered hits.

For each immunofluorescence or immunoblotting experiment, U-2 OS/centrin-GFP cells were grown in 60 mm tissue culture dishes with 2 mL DMEM free of antibiotics. Cells were transfected with 12 μL of 20 μM annealed RNA duplexes using Oligofectamine transfection reagent and treated as above. Knock-down efficiency was monitored by western blot analysis or immunofluorescence microscopy.

Immunoblotting

For immunoblot analyses, cell lysates were prepared by scraping cells into lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate in dH_2O) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, 2 μM vanadate). Lysates were incubated for 1 h with rotating at 4°C and then cleared by centrifugation for 30 min at 13,000 rpm at 4°C. Protein concentrations were determined by the Bradford assay (Biorad Laboratories, Hercules, CA, USA). 30 μg of protein was loaded on a 4-

12% Bis-Tris or 3-8% Tris-Acetate gel (Invitrogen, Carlsbad, CA, USA) and blotted onto a nitrocellulose membrane.

Immunofluorescence Microscopy

For immunofluorescence microscopic analyses, cells grown on coverslips were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, washed in PBS and permeabilized with 1% Triton-X-100 in PBS for 20 min. After blocking in 10% normal donkey serum (Jackson Immunoresearch, West Grove, PA, USA), cells were incubated with primary antibody overnight. The next morning, cells were warmed at 37°C for 2 h, washed in PBS and incubated with Rhodamine Red-conjugated secondary antibody for 2 h and mounted with DAPI. Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

Electron Microscopy

For transmission electron microscopy, the samples were fixed for at least 1 hour in a 2% glutaraldehyde solution buffered with PBS. After washing in three changes of PBS, the samples were placed in a 1% osmium tetroxide solution buffered with PBS for one hour, followed by a series of rinses with ethanol solutions of increasing concentration (50%, 70%, 95%, and 100%). The samples were then placed in a 1:1 mixture of Epon Araldite resin and propylene oxide, and held overnight in a desiccator. The following day, the Epon Araldite and propylene oxide mixture was removed and replaced with 100% Epon Araldite resin. The samples were infiltrated with the resin for an additional 8 h, placed in embedding molds and polymerized for 48 h at 60°C. Serial thin sections were cut using a Reichert-Jung Ultracut E ultramicrotome

and a DDK Diamond knife. Thin sections were picked up on copper grids and stained with 1% uranyl acetate and Reynold's lead citrate. The sections were viewed on a Hitachi H-7100 TEM transmission electron microscope (Hitachi High Technologies America, Pleasanton, CA). Digital images were obtained using an AMT Advantage 10 CCD Camera System (Advanced Microscopy Techniques Corporation, Danvers, MA) and NIH Image software.

Statistical Methods

Student's *t* test for independent samples was used wherever applicable.

4.3 RESULTS

Inhibition of the Proteasome Induces Abnormal Elongation of Daughter Centrioles

To examine the role of proteolysis in centriole biogenesis, we treated U-2 OS cells stably expressing green fluorescent protein (GFP)-tagged centrin (U-2 OS/centrin-GFP), with the proteasome inhibitor Z-L_3VS [154]. Treatment of cells for 72 h with Z-L_3VS was found to induce a significant elongation of centrioles (Figure 17A). Elongated centrioles were typically arranged in a 'flower'-like pattern around a large centrin-GFP dot an described before in Z-L_3VS-treated cells [80]. Occassionally elongated centrioles were found to have a bifurcated end (Figure 1A bottom left panel, arrow). This observation is similar to previous reports when abnormal centriole elongation was induced through prolonged overexpression of the protein CPAP [153, 155, 156]. Z-L_3VS-induced abnormally elongated centrioles were stable and persisted in a significant proportion of cells for up to 72 h after removal of the drug (data not shown).

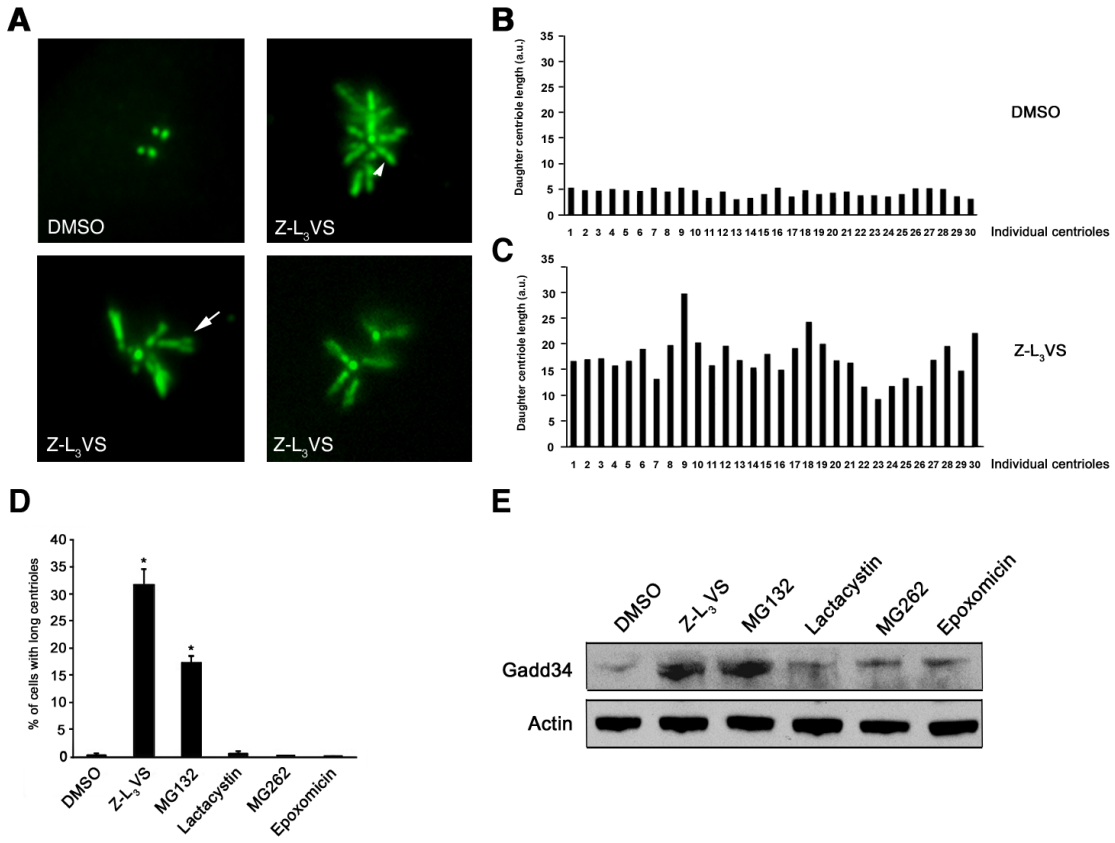

Figure 17. Inhibition of the proteasome stimulates aberrant centriole elongation.

(A) Fluorescence microscopic analysis of U-2 OS cells stably expressing centrin-GFP after either treatment with 0.1% DMSO (top left panel) or 1 μM Z-L_3VS (remaining panels) for 72 h. Arrow indicates a bifurcated distal end of an elongated centriole. Arrowhead points to an example of an elongated centriole with segmentation of the centrin-GFP signal. (B,C) Quantification of centriole length in U-2 OS/centrin GFP cells with abnormally elongated centrioles after treatment with 0.1% DMSO or 1 μM Z-L_3VS for 72 h. Centriole length measurements were performed using ImageJ and expressed as arbitrary units (a.u.). (D) Quantification of the percentage of U-2 OS/centrin-GFP cells containing elongated centrioles after treatment with Z-L_3VS (1 μM), MG132 (1 μM), lactacystin (1 μM), MG262 (0.001 μM) and epoxomicin (0.01 μM) for 72 h. Cells treated with 0.1% DMSO for 72 h were used as controls. All these inhibitor concentrations were associated with at least 50% cell viability. Each bar represents mean and standard error of at least two independent experiments with a minimum of 100 cells counted per experiment. Asterisks indicate statistically significant differences ($p \leq 0.0001$; Student's t test for independent samples). Legend is continued on following page.

(E) Immunoblot analysis of Gadd34 in U-2 OS/centrin-GFP cells after treatment with either 0.1% DMSO or Z-L_3VS (1 μM), MG132 (1 μM), lactacystin (1 μM), MG262 (0.001 μM) and epoxomicin (0.01 μM) for 72 h. All these inhibitor concentrations were associated with at least 50% cell viability. Immunoblot for actin is shown to demonstrate protein loading.

In order to assess the increase in centriole length induced by Z-L_3VS in a semi-quantitative manner, centriole length was measured using the ImageJ software (http://rsbweb.nih.gov/ij/) and expressed in arbitrary units (Figure 17B and 17C). In cells treated with Z-L_3VS, the maximum length of individual centrioles (n=30) was considerably longer and varied widely compared to the shorter and more constant length of individual centrioles in DMSO-treated control cells (n=30), underscoring the intrinsic ability of centrioles to elongate to several times their normal length.

We next tested a panel of different proteasome inhibitors for their ability to induce abnormal centriole elongation using a drug concentration that resulted in a cell viability of at least 50%. Quantification of cells with elongated centrioles revealed a significant 95.8-fold increase in populations treated with 1 μM Z-L_3VS for 72 h (31.6%; $p \leq 0.0001$) when compared to DMSO-treated controls (0.33%; Figure 17D). A significant increase of cells with elongated daughter centrioles, although to a lesser extent, was also detected in cells treated with the proteasome inhibitor MG132 (17.3%; $p \leq 0.0001$) but not in cells treated with MG262, lactacystin, or epoximicin (Figure 17D).

To confirm the role of proteolysis in centriole length control, we repeated these experiments with the proteasome inhibitors which did not initially induce centriole elongation at higher drug concentrations, albeit at the expense of cell viability. Cells treated with a ten-fold increase in concentration of MG262, a five-fold increase in concentration of epoxomicin, and a

ten-fold increase in concentration of lactacystin exhibited elongated centrioles in 2.0%, 3.7% and 2.3% of cells, respectively (data not shown).

Now that we demonstrated proteasome inhibition in general leads to abnormal centriole elongation, we next sought to determine why Z-L_3VS and MG132 were much more potent inducers of abnormal daughter centriole elongation compared to the other proteasome inhibitors, in particular lactacystin. One explanation for this difference could be the efficacy and timing of proteasome inhibition achieved in U 2-OS/centrin-GFP cells. We addressed this by performing immunoblot analysis of Gadd34, a robust marker of 26S proteasome activity [157], following treatment with our panel of proteasome inhibitors at the original concentration leading to greater than 50% cell viability. Only treatment with Z-L_3VS or MG132 promoted accumulation of Gadd34 at 72 h post-inhibitor treatment, the time point at which we assessed daughter centriole length (Figure 17E). This suggests that sustained inhibition of the 26S proteasome, among other factors (see Discussion), may play a role in Z-L_3VS- or MG132-induced centriole elongation.

To determine that the elongated centrin-positive structures observed in Z-L_3VS-treated U-2 OS/centrin-GFP cells were in fact daughter centrioles, we examined Z-L_3VS-treated cells by ultrathin serial section electron microscopy (EM). As shown in Figure 18A, the elongated structures observed were bona fide daughter centrioles as indicated by the lack of detectable appendages.

While analyzing elongated daughter centrioles, we observed regions of the elongated daughter that contained less intense centrin staining (Figure 18A, arrowhead). This finding raised the question whether elongated daughters consist of centrin-containing segments or continuously elongated centriolar structures. To answer this question, we specifically analyzed these regions of abnormally elongated daughter centrioles. As shown in Figure 18B, we found microtubules

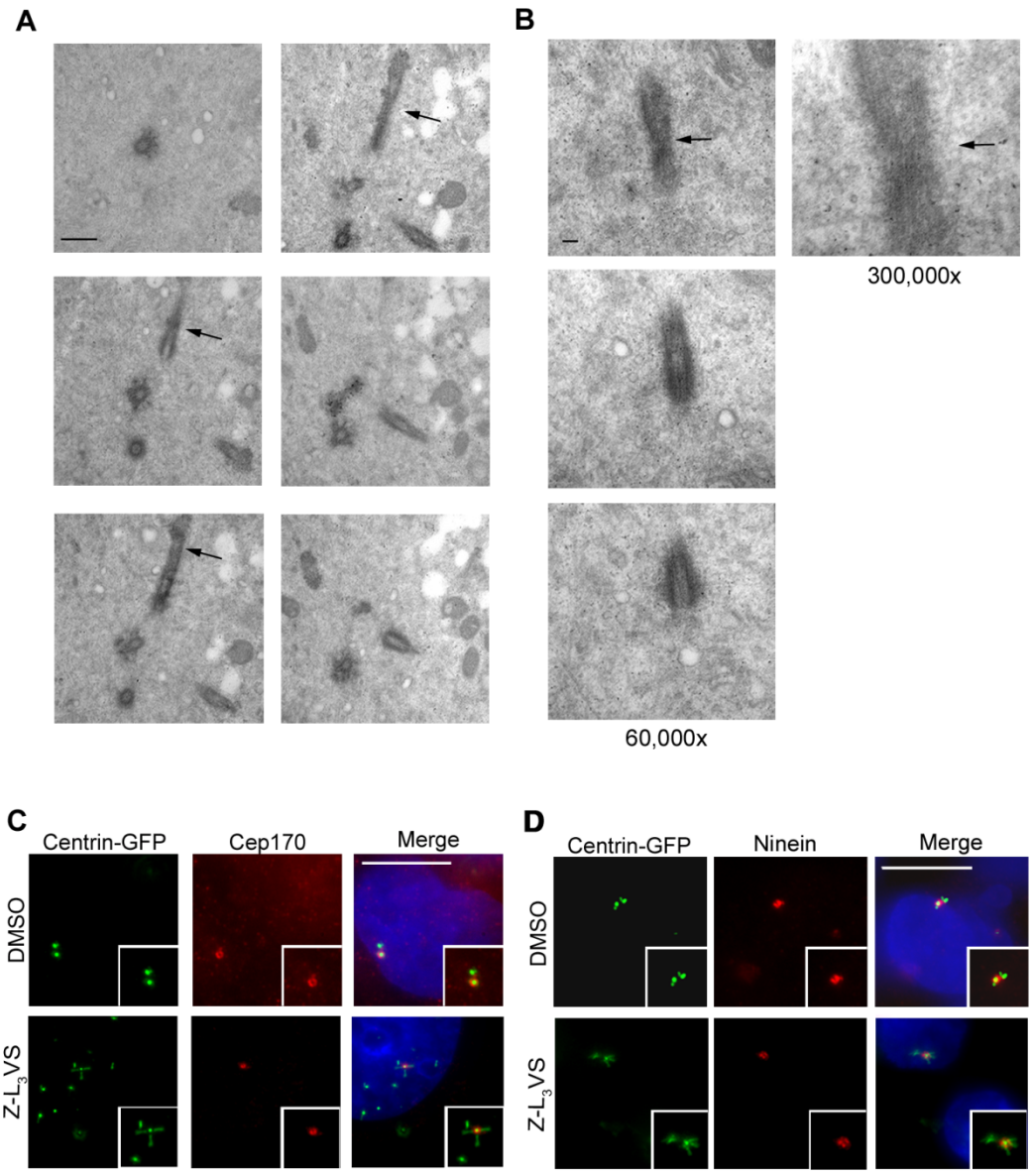

Figure 18. Inhibition of the proteasome stimulates elongation of daughter but not maternal centrioles.

(A) Consecutive serial-section electron microscopic analysis of an abnormally elongated centriole after Z-L_3VS treatment of U-2 OS/centrin-GFP cells. Arrows point to an elongated daughter centriole. Scale bar indicates 500 nm. (B) Consecutive serial-section electron microscopic analysis of a elongated daughter centriole after 72 h treatment of U-2 OS/centrin-GFP cells with Z-L_3VS. Note the less electron dense area and the continuous microtubules that span this region (arrows). Scale bar indicates 100 nm. (C,D) Immunofluorescence microscopic analysis for Cep170 (C) and ninein (D) in U-2 OS/centrin-GFP cells after a 72 h treatment with the proteasome inhibitors Z-L_3VS (1 μM) or 0.1% DMSO as control. Nuclei stained with DAPI. Scale bar indicates 10 μm.

spanning this electron dense region by ultrathin serial section EM, underscoring that elongated daughter centrioles consist of elongated centriolar microtubules and not discrete centrin-containing segments.

To further substantiate that these elongated structures represent elongated daughter centrioles, we performed immunofluorescence staining of Cep170 and ninein, two markers of mature maternal centrioles [77, 158]. Elongated centrioles were grouped around a central, maternal centriole and did not co-localize with either Cep170 or ninein, indicating that elongated centrin positive structures were indeed daughter centrioles (Figure 18C and 18D).

Taken together, our results suggest that Z-L_3VS and MG132 treatment leads to an abnormal elongation of daughter centriolar microtubules and that normal daughter centriole length is not a result of structural constraints but is controlled by proteolysis.

Abnormal Elongation of Daughter Centrioles Involves Several Centrosomal Proteins

In order to determine the underlying mechanisms of abnormal elongation of daughter centrioles, we performed an siRNA screen focusing on 127 proteins known to be associated with centrosomes [152] (data not shown). Twenty-four hours after siRNA-treatment of U-2 OS/centrin-GFP cells, elongation of daughter centrioles was induced by treatment of cells with the proteasome inhibitor Z-L_3VS for 72h. Protein depletion was assessed by immunoblot analysis or immunofluorescence microscopy (data not shown). Of 127 proteins analyzed, depletion of nine centrosomal proteins reproducibly changed the percentage of cells containing elongated centrioles when normalized to Z-L_3VS-treated control siRNA transfected cells, suggesting that the target proteins may be involved in regulating centriole elongation (Figure

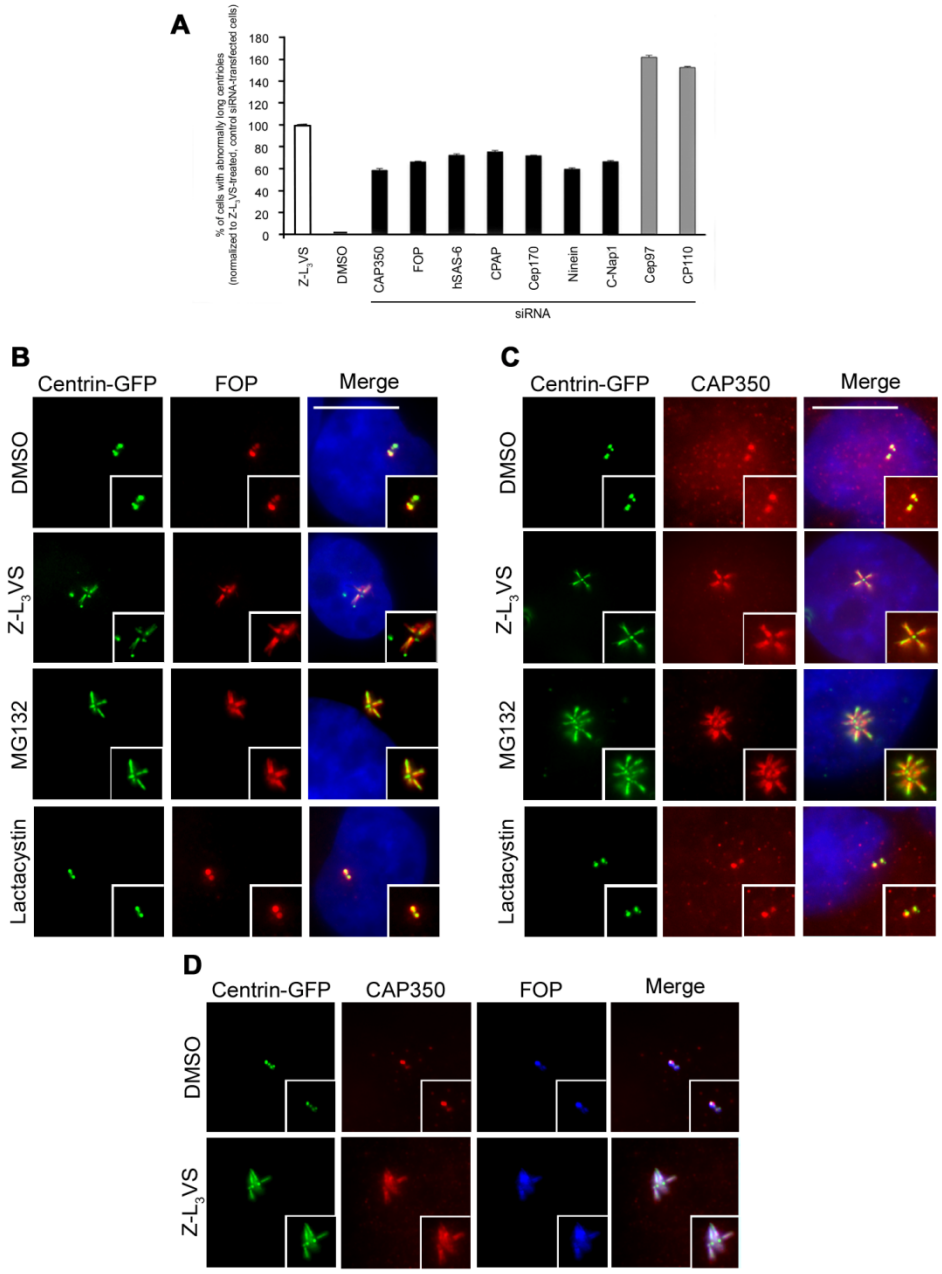

Figure 19. An siRNA screen to identify centrosomal proteins involved in Z-L_3VS-induced abnormal daughter centriole elongation.

(A) U-2 OS-centrin/GFP cells were transfected for 24 h with control or siRNA duplexes targeting 127 known centrosomal proteins [152] followed by a 72 h exposure to the proteasome inhibitor Z-L_3VS (1 μM) or 0.1% DMSO as control. Cells were analyzed by fluorescence microscopy to determine the proportion of cells that contained abnormally long daughter centrioles. The bar graph shows negative (black bars) and positive (grey bars) regulators of Z-L_3VS-induced daughter centriole elongation. Legend is continued on following page. Each bar represents the

proportion of cells containing elongated centrioles when normalized to Z-L_3VS-treated cells transfected with control siRNA duplexes (set to 100%). Mean and standard error of at least two independent experiments with a minimum of 100 cells counted per experiment is shown. (B,C) Immunofluorescence microscopic analysis for FOP (B) and CAP350 (C) in U-2 OS/centrin-GFP cells after a 72 h treatment with the proteasome inhibitors Z-L_3VS, MG132, or lactacystin (all 1 μM). Treatment with 0.1% DMSO was used as control. Scale bar indicates 10 mm. (D) Co-immunofluorescence microscopic analysis for FOP and CAP350 in U-2 OS/centrin-GFP cells after a 72 h treatment with the proteasome inhibitors Z-L_3VS (1 μM) or 0.1% DMSO. Nuclei stained with DAPI.

19A). Depletion of seven proteins, FOP, CAP350, CPAP, hSAS6, Cep170, ninein, and C-Nap1 led to a decrease in the number of cells that contained elongated daughter centrioles, whereas depletion of two proteins, Cep97 and CP110, led to an increase in the number of cells that contained elongated daughter centrioles (Figure 19A).

Depletion of two proteins implicated in microtubule anchoring and stability, FOP and CAP350 [159-161], significantly reduced the number of cells containing abnormally elongated daughter centrioles to 50.7% and 54.7%, respectively, of control siRNA transfected Z-L_3VS-treated cells. Furthermore, depletion of Cep97 and CP110, two proteins that were previously shown to suppress the formation of cilia [145], led to an increase in the number of cells that contained long centrioles to 162.7% and 153.3%, respectively of control siRNA transfected Z-L_3VS-treated cells. This is in line with previous results suggesting that CP110 plays a negative regulatory role in centriole elongation [153, 156].

Depletion of hSAS-6, a structural protein which is required for daughter centriole synthesis [88], decreased the percentage of Z-L_3VS-treated cells which contained long daughter centrioles to 76% of control siRNA Z-L_3VS-treated cells (Figure 19A). In addition, depletion of CPAP, a protein that has previously been shown to play a role in centriole elongation [153, 155, 156], also decreased the percentage of Z-L_3VS-treated cells with long daughter centrioles to

73.2% of control siRNA Z-L_3VS-treated cells (Figure 19A). Remarkably, depletion of proteins implicated either in maintaining centrosome cohesion or associated with maternal centriole appendages, C-Nap1 [162], ninein [158], and Cep170 [77], also caused a reduction in the number of cells containing long centrioles to 67.1%, 60.3%, and 72.6%, respectively, compared to control siRNA transfected Z-L_3VS-treated cells (Figure 19A).

Taken together, these results suggest that Z-L_3VS-induced abnormal centriole elongation involves known positive and negative microtubule regulatory proteins as well as proteins involved in centrosome cohesion and microtubule anchoring.

Z-L_3VS Alters the Expression of Several Centriolar Proteins Involved in Length Control

Next, we asked whether Z-L_3VS or MG132-induced abnormal daughter centriole elongation was associated with alteration of the localization and/or abundance of the nine proteins we identified in our siRNA screen. First, we analyzed the localization of the two major microtubule stabilizing proteins identified in our screen, FOP and CAP350. Immunofluorescence microscopy of FOP in DMSO-treated control cells, showed FOP co-localizing with centrioles (Figure 19B, top panel), as previously noted [159, 161]. When cells were treated with Z-L_3VS or MG132, staining for FOP was found to co-localize along elongated daughter centrioles (Figure 19B, middle panels). However, when cells were treated with lactacystin, a proteasome inhibitor that is less potent to induce abnormal elongation of daughter centrioles, FOP localization was similar to that of DMSO-treated control cells (Figure 19B, bottom panels).

Immunofluorescence microscopic analysis of CAP350 in DMSO-treated control cells showed CAP350 localization to both mother and daughter centrioles as previously reported [159, 161] (Figure 19C, top panel). When CAP350 was analyzed after Z-L_3VS or MG132-treatment,

we detected CAP350 co-localizing along elongated daughter centrioles, similar to the localization of FOP after treatment with these proteasome inhibitors (Figure 19C, middle panels). When cells were treated with lactacystin, CAP350 localization was comparable to that of DMSO-treated control cells (Figure 19C bottom panels). We confirmed the similar localization of FOP and CAP350 along elongated daughters by performing co-immunofluorescence microscopic analysis following Z-L_3VS-treatment (Figure 19D).

To assess the accumulation of FOP and CAP350 protein, respectively, an immunoblot analysis of whole cell extracts from cells treated for 72 h with our panel of proteasome inhibitors was performed. Z-L_3VS or MG132-treatment, which both induce abnormal elongation of daughter centrioles, resulted in the accumulation of both FOP and CAP350 to significantly higher levels than in DMSO-treated controls (Figure 20A). The proteasome inhibitors that were unable to induce abnormal daughter centriole elongation at concentrations associated with at least 50% cell viability, did not demonstrate an accumulation of either FOP or CAP350 compared to DMSO-treated control cells (Figure 20A).

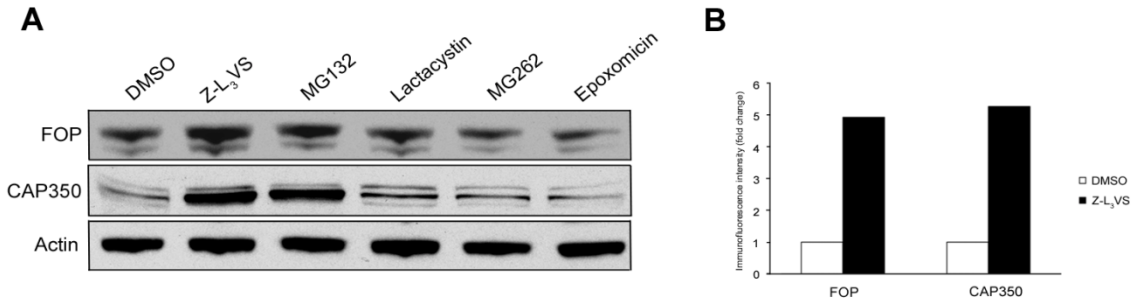

Figure 20. Stabilization of FOP and CAP350 protein by both Z-L_3VS and MG132 treatment.

(A) Immunoblot analysis of FOP and CAP350 in U-2 OS/centrin-GFP cells after treatment with either 0.1% DMSO or proteasome inhibitors as indicated for 72 h. Immunoblot for actin is shown to demonstrate protein loading. Note accumulation of both CAP350 and FOP in Z-L_3VS-treated cells. Legend is continued on the following page.

(B) Quantification of the immunofluorescence intensity of FOP and CAP350 in U-2 OS/centrin GFP cells with abnormally elongated centrioles after treatment with 0.1% DMSO or 1 μM Z-L_3VS for 72 h. Fluorescence intensity measurements were performed using ImageJ.

To corroborate that accumulation of FOP and CAP350 was in fact occurring at the centrosome, we performed semi-quantitative analysis of centrosomal protein levels by immunofluorescence analysis using ImageJ software (Figure 20B). We found a 4.9-fold increase in FOP and a 5.3-fold increase in CAP350 protein levels in Z-L_3VS-treated cells versus DMSO-treated control cells. Furthermore, we analyzed individual centriole length upon transfection of siRNA duplexes targeting either FOP or CAP350, followed by Z-L_3VS-treatment, as we had previously done in Figure 17B and 17C. We found that the maximum length of individual daughter centrioles was overall reduced compared to controls although the length of individual abnormally elongated daughter centrioles still varied widely (data not shown). Together, these results suggest that protein stabilization of both FOP and CAP350 through proteasome inhibition contributes to abnormal elongation of daughter centrioles.

We next examined the localization of CPAP, a known microtubule-interacting protein that has recently been implicated in centriole length control, after treatment with proteasome inhibitors (Fig. 21). In DMSO-treated control cells, we saw CPAP localizing to both mother and daughter centrioles (Figure 21, top panel), as previously seen [153, 155, 156]. When we treated cells with Z-L_3VS, CPAP co-localized with the centrin signal of elongated daughter centrioles (Figure 21, middle panel). However, when cells were treated with the proteasome inhibitor lactacystin at a concentration that did not lead to centriole elongation, CPAP localization was analogous to DMSO-treated control cells (Figure 21, bottom panel). This suggests that treatment

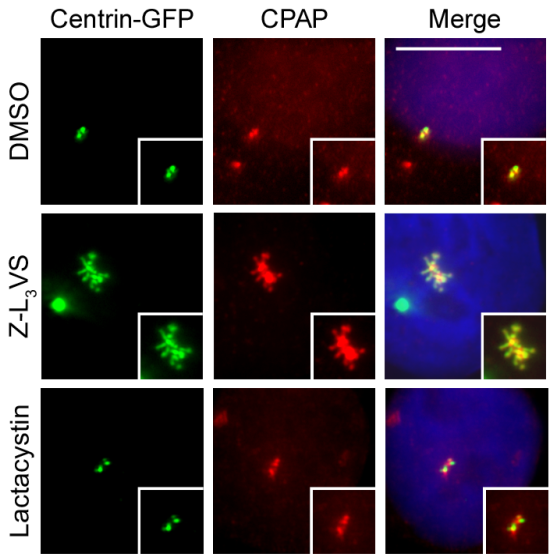

Figure 21. Alteration of CPAP localization following Z-L_3VS treatment.

Immunofluorescence microscopic analysis of CPAP in U-2 OS/centrin-GFP cells after treatment with 1 μM Z-L_3VS for 72 h or 0.1% DMSO as control. Nuclei stained with DAPI. Scale bar indicates 10 μm.

with the proteasome inhibitor Z-L_3VS leads to an accumulation of CPAP at elongated daughter centrioles.

Although depletion of C-Nap1, hSAS-6, Cep170, and ninein decreased Z-L_3VS mediated abnormal daughter centriole elongation in our siRNA screen, the localization of these proteins did not change in cells treated with any of our proteasome inhibitors (Figures 22). This suggests that, although treatment with Z-L_3VS does not promote a significant change in phenotype of these proteins, the presence of C-Nap1, hSAS-6, Cep170, and ninein is necessary in order to promote Z-L_3VS mediated abnormal daughter centriole elongation.

We then determined the localization of CP110 and Cep97, two proteins whose depletion we found to increase the percentage of Z-L_3VS treated cells that contained long daughter centrioles. Immunofluorescence microscopic analysis of CP110 and Cep97 in DMSO-treated control cells showed centriolar localization of both proteins (Figure 23A and 23B, top panels) in

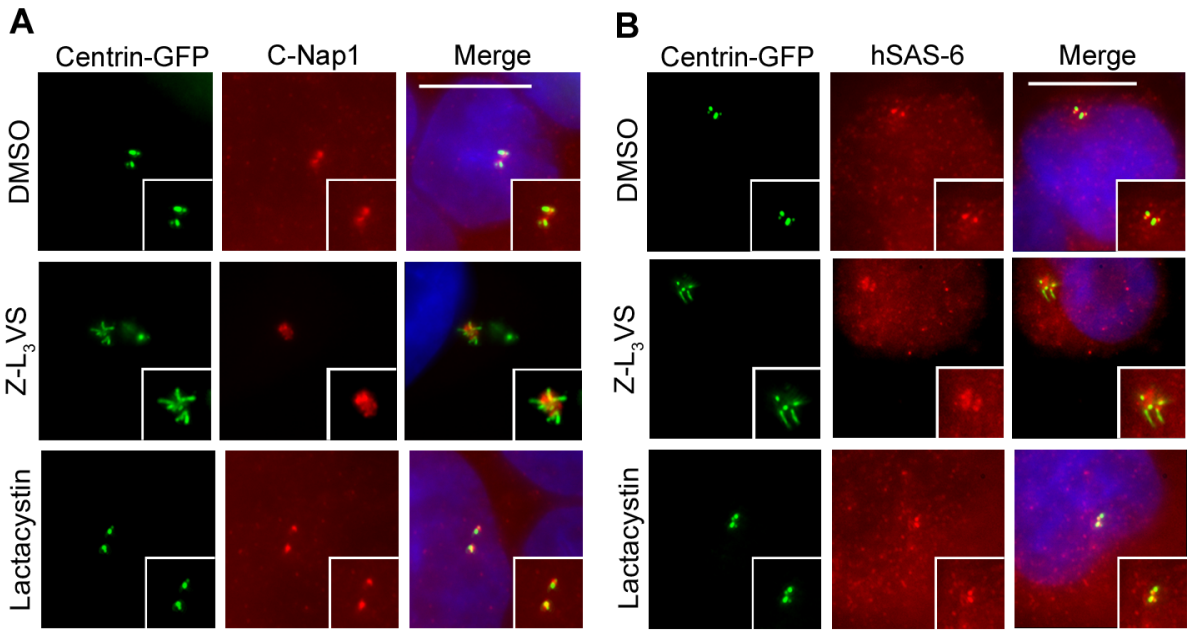

Figure 22. Expression C-Nap1 and hSAS-6 following Z-L_3VS treatment.

(A,B) Immunofluorescence microscopic analysis for C-Nap1 (A) or hSAS-6 (B) in U-2 OS/centrin-GFP cells after a 72 h treatment with 1 µM the proteasome inhibitor Z-L_3VS or with 1 µM lactacystin. Treatment with 0.1% DMSO was used as control. Nuclei stained with DAPI. Scale bar indicates 10 µm.

accordance with previously published findings [87, 145, 156, 163]. When cells were treated with Z-L_3VS to induce abnormally elongated daughter centrioles, both CP110 and Cep97 localized to the tips of elongated daughters (Figure 23A and 23B, middle panel). Treatment of cells with lactacystin at concentrations that did not induce elongated daughter centrioles, resulted in a similar CP110 and Cep97 localization pattern as detected DMSO-treated controls (Figure 23A and 23B, bottom panels).

Taken together, Z-L_3VS-induced abnormal daughter cell elongation was associated with prominent changes in FOP, CAP350 and CPAP protein abundance and changes in localization at long daughter centrioles. Not all proteins identified in our siRNA screen followed this pattern

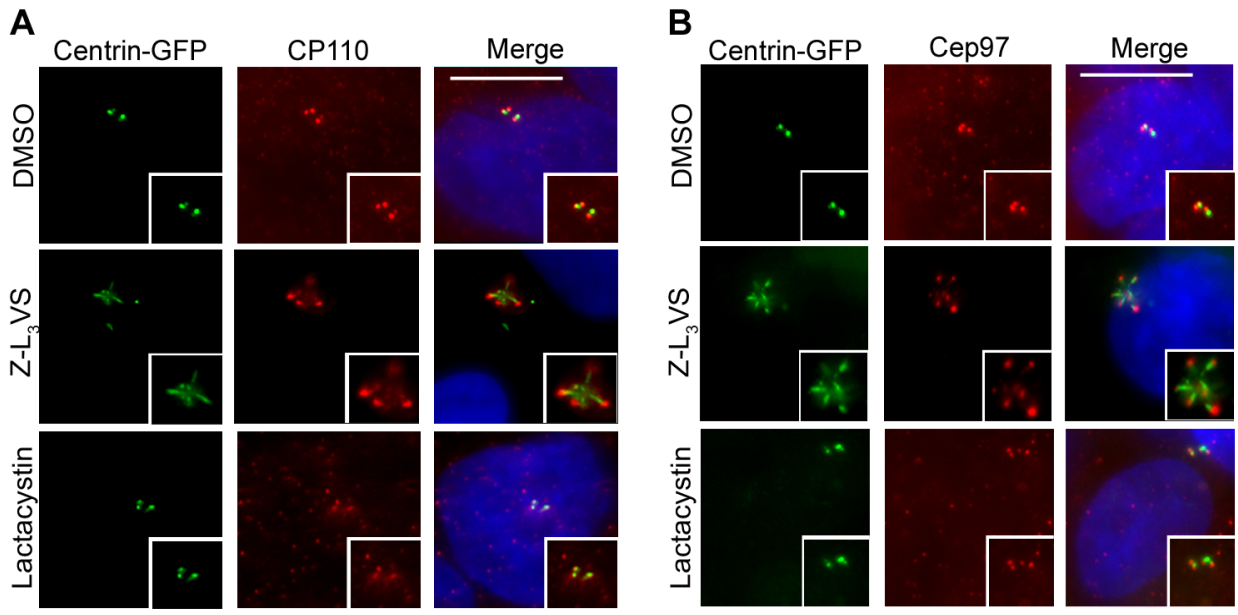

Figure 23. CP110 and Cep97 are retained at the tips of elongated daughter centrioles induced by Z-L_3VS.

(A,B) Immunofluorescence microscopic analysis of CP110 (A) and Cep97 (B) in U-2 OS/centrin-GFP cells after a 72 h treatment with 1 μM of the proteasome inhibitors Z-L_3VS or 1μM lactacystin. Treatment with 0.1% DMSO was used as control. Note the localization of both CP110 and Cep97 to the distal ends of elongated daughter centrioles. Nuclei stained with DAPI. Scale bar indicates 10 μm.

although most proteins showed an accumulation at centrioles by immunofluorescence microscopy (C-Nap1, hSAS-6, CEP170, ninein, Cep97; data not shown). Clearly, Z-L_3VS-induced abnormal centriole elongation was not due to displacement of CP110 and Cep97 from the distal tips of daughter centrioles (Fig. 23). However, the lack of increased CP110 protein levels in Z-L_3VS- compared to DMSO-treated cells, as measured by immunofluorescence analysis, is in agreement with the role of CP110 as a negative regulator of centriole elongation [148-150]. Taken together, these results suggest that some proteins may play a direct role in aberrant microtubule elongation along the length of daughter centrioles whereas others may not function directly at daughter centrioles but are yet required for this process.

4.4 DISCUSSION

The present report provides unexpected insights into the regulation of daughter centriole elongation and underscores the role of proteolysis in this process. We show that daughter centriole elongation is not controlled by structural constraints but is a highly regulated process that involves several more proteins than CPAP and CP110, which have previously been implicated in the regulation of centriole elongation.

Since we only detected abnormally long daughter centrioles under our assay conditions, our findings suggest that mother and daughter centriole elongation are, to a certain degree, separable processes and can be distinguished by proteolytic inhibition. The basis for this differential regulation is unknown but it is possible that cell cycle-dependent events play a role. Daughter centriole elongation predominantly occurs during S and G2 phases of the cell division cycle. Hence, the treatment with proteasome inhibitors Z-L_3VS or MG132 may render cells competent for aberrant daughter centriole elongation through prolongation of a cell cycle stage that is permissive for this process. In line with this notion is our previous finding that Z-L_3VS-treated cells accumulate in G2 [80]. However, a cell cycle arrest per se is not sufficient to induce abnormal daughter centriole elongation since cells treated with epoxomycin contained a significant proportion of cells that were arrested in G2 phase of the cell division cycle (data not shown), but lacked the ability to produce abnormally long daughter centrioles (Fig. 17D).

When we first tested different proteasome inhibitors for their ability to induce abnormal centriole elongation, we used a drug concentration that resulted in a cell viability of at least 50%. We repeated these experiments with higher concentrations of proteasome inhibitors and we found that, in principle, all proteasome inhibitors used were able to induce abnormally long centrioles, albeit associated with reduced cell viability and in an overall lower percentage of cells

that survived the treatment. Remarkably, abnormally elongated centrioles were virtually absent in DMSO-treated control cells. Collectively, these results confirm that proteasome inhibition leads to abnormal centriole elongation, however, to different extents.

To determine why Z-L_3VS and MG132 were much more potent inducers of abnormal daughter centriole elongation compared to the other proteasome inhibitors, in particular lactacystin, we tested their proteasome inhibitory activities by immunoblotting for Gadd34 [157]. We found that only treatment with Z-L_3VS or MG132 promoted a sustained inhibition of the 26S proteasome at 72 h, which may explain why Z-L_3VS and MG132, probably in conjunction with other characteristics (see below), are the most potent inducers of aberrant daughter centriole elongation.

Although all proteasome inhibitors tested here have been shown to inhibit 26S proteasome activity, several of our inhibitors, most notably Z-L_3VS and MG132, also have non-proteasomal activities against cellular proteases. In contrast, other inhibitors such as lactacystin and epoxomicin are more potent and specific inhibitors of the 26S proteasome with less non-proteasomal inhibitory activities. Nonetheless, these non-proteasomal activities may become more prominent at higher inhibitor concentrations and may play a role in the small amount of centriole elongation seen when inhibitors that did not score in our initial screen were used at higher concentrations. These results may furthermore suggest that non-proteasomal functions, specifically inhibition of cellular proteases, may play an important but currently underappreciated role in the regulation of centriole elongation.

To determine what proteins were involved in abnormal daughter centriole elongation induced by Z-L_3VS, we utilized an siRNA screen based on a previous study, in which 127 centrosomally associated proteins were identified [152]. It is important to note that by using

targets identified in this previous study, we may be excluding centrosomal proteins not identified in this screen which could potentially play a role in centriole elongation control.

We did not see a complete abolishment of abnormal centriole length in our siRNA screen and believe this is explained by the discrepancy between the rapid effects of proteasome inhibition on centriole biogenesis and the slower kinetics of maximum siRNA efficacy. Z-L_3VS-associated alterations of centriole biogenesis become visible already at 6 h after treatment and affect approximately 25% of cells after 24 h of treatment (our own unpublished results). It is generally accepted that siRNA-mediated knock-down of protein expression usually results in maximum protein depletion at approximately 48-72 h post-transfection. The residual extra long daughter centrioles that we observed are likely to have formed prior to complete knock-down of the targeted proteins.

In addition, the abnormal centriole elongation phenotype reported here mostly arises in the context of centriole overduplication, specifically centriole multiplication, during which a single maternal centriole nucleates the concurrent formation of multiple daughter centrioles [80]. These supernumerary centrioles were very stable and persisted for prolonged time intervals [80]. In line with this notion, we found that Z-L_3VS-induced abnormally elongated centrioles persisted in a significant proportion of cells for up to 72 h after removal of the drug. It is hence possible that abnormally elongated daughter centrioles that form in response to proteasome inhibition are particularly stable and less dynamic, a possibility that is currently under investigation.

Nonetheless, results from this siRNA screen confirm and extend a number of previous studies. Recently, overexpression of CPAP was shown to induce the abnormal elongation of both mother and daughter centrioles [153, 155, 156]. CPAP protein level was shown to be cell cycle regulated through the action of the anaphase-promoting complex/cyclosome (APC/C) and the

26S proteasome [153]. Our screen confirms this role of CPAP in centriole elongation by showing that CPAP depletion reduces the number of cells which exhibit centriole elongation following Z-L_3VS-treatment.

CPAP overexpression was found to synergize with depletion of another centriole component, CP110, in the formation of extra long centrioles [153, 155]. CP110 is a distal-end capping protein and knock-down of this protein alone can leads to centriole elongation and formation of primary cilia [145]. These results have suggested a simple model in which CPAP and CP110 act as positive and negative regulators, respectively, of centriole elongation. In line with this model, we found that depletion of either CP110, or a CP110 interacting partner Cep97, enhanced Z-L_3VS-mediated daughter centriole elongation.

However, our results significantly extend these previous studies by showing that a number of additional proteins besides CPAP and CP110 are involved in centriole length control. We found that FOP and CAP350 were also necessary for abnormal daughter centriole elongation, and significantly accumulate in Z-L_3VS or MG132 treated cells. FOP and CAP350 have been shown to interact and form a complex at the centrosome, with CAP350 necessary for FOP localization [159]. The FOP-CAP350 complex was shown to play a role in microtubule anchoring at the centrosome. However, both proteins also contain specific domains, in the case of FOP a LisH domain and of CAP350 a CAP-gly domain, which have been hypothesized to be involved in microtubule stability and dynamics [159, 164-166]; [160, 161]. Recently it was shown that neither FOP nor CAP350 depletion prevented initiation of procentriole synthesis, but that CAP350 was necessary for procentriole elongation to occur [161]. In line with this, we analyzed the individual length of daughter centrioles upon transfection of siRNA duplexes targeting either FOP or CAP350, followed by Z-L_3VS-treatment, and found that the maximum

length of individual daughter centrioles was overall reduced compared to controls although the length of individual abnormally elongated daughter centrioles still varied widely (data not shown). The striking change in the expression pattern of both FOP and CAP350 shown here, along with the reduction of individual centriole length found in cells depleted of these two proteins, strengthens the hypothesis that these two proteins function to stabilize growing centriolar microtubules thereby promoting Z-L_3VS and MG132-induced daughter centriole elongation.

In addition, we show that hSAS-6, a structural centriolar component that is essential for daughter centriole synthesis [87, 88], is also necessary for abnormal centriolar elongation induced by Z-L_3VS. Moreover, C-Nap1 a protein known to play a role in maintaining centrosome cohesion, as well as Cep170 and ninein, both proteins known to associate with maternal centriolar appendages, were also found to play a role in centriole length control. Depletion of Cep170 may interfere with microtubule-dependent trafficking to the centrosome [77], while depletion of ninein may result in the loss of microtubule anchoring at the centrosome [167]. Our results hence suggest that centrosome cohesion and microtubule-dependent processes are necessary for abnormal centriole elongation. Support for this notion comes from previous work suggesting that both centriolar and pericentriolar components are necessary for centriole assembly, possibly by concentrating the recruitment of components necessary for daughter centriole synthesis around the maternal centriole [74, 168]. We believe that the accumulation of hSAS-6, C-NAP1, CEP170 and ninein seen by immunofluorescence analysis, further supports the role of these proteins in Z-L_3VS-induced daughter centriole elongation. Collectively, these results and results by others [88, 148] confirm and extend the notion that key regulators of Z-L_3VS-induced daughter centriole elongation are controlled by proteolysis.

In conclusion, we provide evidence that daughter centriole elongation is not limited by structural constrains but rather regulated by proteolysis. Our results suggest that maintaining the balance between positive and negative regulatory proteins is key for daughter centriole elongation control. We show that a number of additional proteins besides CPAP and CP110 are involved in this process including proteins known to play a role in centrosome cohesion and microtubule anchoring. This report illustrates the complex circuitry of centriolar proteins that regulate centriole biogenesis including daughter centriole length control and reveals a link between control of centriole elongation and the suppression of the cilia assembly pathway highlighting the critical role of proteolysis in this process.

5.0 GENERAL DISCUSSION

Persistent infection with high-risk HPV is the major driving force in the development of cervical cancer. Aneuploidy is frequently found in pre-malignant high-risk HPV-associated lesions and is a critical factor for malignant progression. The high-risk HPV-16 E7 oncoprotein has been shown to promote centrosome abnormalities and numerical chromosomal instability thereby increasing the risk for aneuploidy and malignant progression, through mechanisms that are incompletely understood [169]. The purpose of this study was to answer several key questions: (1) How does proteolysis regulate centriole duplication control? (2) Through what mechanisms does HPV-16 E7 expression rapidly promote centriole multiplication? (3) Besides centriole duplication control, does proteolysis control other aspects of centriole biogenesis? This chapter will discuss our findings and highlight how HPV-16 E7 exploits the tight regulation of PLK4 protein levels by SCF ubiquitin ligase complexes to promote centriole multiplication. Finally, we will describe how proteolysis plays a major role in other aspects of centriole biogenesis and outline future studies to expand upon this role.

5.1 HOW DOES PROTEOLYSIS REGULATE CENTRIOLE DUPLICATION CONTROL?

The discovery that inhibition of the proteasome strongly induced centriole multiplication suggested that ubiquitin-mediated proteolysis was important in maintaining normal centriole duplication control [80]. Here, we show that CUL1, the core component of SCF-ligase complexes, localizes to maternal centrioles which function as platforms for oncogene-induced centriole overduplication (Chapter 2; [134]). We show through siRNA-mediated depletion of CUL1 and transfection of a dominant-negative CUL1 construct that SCF-E3 ubiquitin ligase activity is necessary for restraining centriole multiplication. Further experiments revealed that overexpression of cyclin E/CDK2 mediates the aberrant recruitment of PLK4 to maternal centrioles. However, endogenous levels of PLK4 were not sufficient to induce centriole multiplication and a concomitant increase in PLK4 protein levels was necessary for this event to occur. Subsequently, PLK4 protein was found to be degraded by the proteasome and regulated in a PLK4 kinase activity-dependent manner by cyclin E/CDK2. This regulation of PLK4 by the important cell cycle mediators CDK2 and cyclin E may represent a cellular failsafe mechanism to limit normal centriole biogenesis to once and only once per cell division cycle (Chapter 2; [134]). We furthermore show that CUL1 contributes to the control of PLK4 protein levels and active SCF complexes are necessary for this regulation. This was evidenced by accumulation of excess endogenous PLK4 at maternal centrioles following ectopic expression of a dominant-negative CUL1 (DN-CUL1) construct. In addition, ectopic expression of DN-CUL1 along with CDK2 and cyclin E abrogated the activity-dependent degradation of PLK4. Overexpression of CAND1, an inhibitor of SCF-ligase complexes, led to an enhancement in centriole multiplication over that induced by PLK4 overexpression alone. CAND1 overexpression was shown to stabilize

PLK4 protein levels (Chapter 2; [134]). Collectively, these results underscore the importance of SCF E3 ligase mediated proteolysis in restraining centriole biogenesis and maintaining normal centriole duplication control.

It is important to note that ubiquitin-mediated proteolysis is not the only mechanism which controls PLK4 protein stability. The PLK4 protein coding sequence has been reported to contain a PEST domain implicated in the mediation of rapid protein degradation by intracellular proteases [170, 171]. Further experiments to determine if PEST-domain mediated PLK4 degradation may play a role in controlling centriole duplication processes are required and are currently underway.

How is PLK4 recognized for SCF-mediated proteolytic degradation?

There are over 70 known human F-box proteins which control the substrate specificity of SCF-ligase complexes [128]. Most substrates require phosphorylation to interact with the F-box protein of an SCF complex. Phosphorylation allows for substrate discrimination by SCF-ligases due to the specific recognition of phosphorylated motifs (phosphodegrons) [172]. A phosphodegron is one or a series of phosphorylated residues on a substrate that directly interacts with a protein–protein interaction domain in an E3 ubiquitin-ligase, such as an F-box containing protein [173].

Several studies performed in *Drosophila melanogaster* cells report Slimb as the F-box protein which targets PLK4 for degradation [98, 99]. Drosophila PLK4 was found to contain a specific phosphodegron motif, conserved in higher eukaryotes, including humans, which mediates an interaction with Slimb. When either the phosphodegron of *D. melanogaster* PLK4 was mutated or Slimb was knocked-down by siRNA, PLK4 protein levels accumulated [98].

Despite this, efforts to define a role for β-TrCP, the mammalian homolog of Slimb, in human cells had inconsistent results [174, 175].

We have performed an siRNA-mediated screen in U-2 OS/centrin-GFP cells of all known human F-box proteins to assay for induction of centriole multiplication. The goal of this screen was to determine the F-box responsible for PLK4 recognition and subsequent degradation in human cells. Although we failed to observe centriole multiplication following knock-down of β-TrCP, we did identify 12 other F-box proteins whose knock-down led to a significant increase in centriole multiplication over control siRNA treated cells. This suggests that PLK4 protein stability and recognition may be regulated by several different F-box proteins. Nonetheless, β-TrCP1$^{-/-}$ mice do exhibit lengthened mitosis, centrosome overduplication, multipolar metaphase spindles and misaligned chromosomes [132]. However, β-TrCP recognizes several key cell cycle regulators, such as claspin, CDC25a and Wee1, suggesting that this mouse model may present confounding cell cycle dysregulation. The coincidence of aberrant centrosome numbers and nuclear abnormalities observed in this model may be due to centrosome accumulation and not genuine centrosome overduplication

PLK4 is known to autophosphorylate and our present study, as well as several previous reports, suggests that autophosphorylation may influence the proteolytic degradation of PLK4 (Chapter 2; [98, 99, 134]). This autophosphorylation may induce a conformational change or prime a phosphodegron to promote PLK4 degradation. In line with this hypothesis, PLK4 was recently found to contain a 24 amino-acid (a.a.) autophosphorylation domain containing 13 phospho-residues [174, 175]. Part of the putative PEST domain, previously proposed to destabilize PLK4, and the β-TrCP phosphodegron motif are contained within this region. Deletion of either the 24 a.a. autophosphorylation region or the kinase domain, dramatically

stabilized PLK4 protein levels [174]. When both phosphorylation sites of the β-TrCP phosphodegron motif were mutated to alanine, β-TrCP was unable to bind the mutated PLK4 construct. Intriguingly, this mutated protein was still able to be ubiquitinated and was only slightly more stable than wild-type PLK4 [174]. Thus, β-TrCP may play only a minor role in the ubiquitin-mediated degradation of PLK4 in human cells underscoring the potential role of either multiple F-box proteins, alternative E3 ubiquitin ligases or cellular proteases in the regulation of PLK4 stability.

Complex proteolytic regulation is seen for key cell cycle regulators such as cyclin E, which is recognized for degradation by more than one F-box protein [176, 177]. Our results suggest that CUL1 controls multiple aspects of PLK4 regulation including both the baseline protein level and its activity-dependent degradation (Chapter 2; [134]). Recognition of active and inactive pools of PLK4 is likely to be mediated by multiple F-box proteins and/or multiple CRLs.

Only a handful of downstream PLK4 targets have been identified in human cells including the transcription factor Hand1 and the phosphatase CDC25C. However, the role of these proteins in centriole biogenesis remains to be determined. Discovering downstream PLK4 targets relevant to centriole biogenesis and determining how these proteins are affected by both overexpression and depletion of PLK4 may also help in determining how PLK4 is regulated.

Elucidating the exact role of CRL complexes in mediating centriole duplication control will provide invaluable information about the regulatory control of centrosomal proteins and perhaps identify new proteins which may be therapeutic targets for the prevention of genomic instability and malignant progression in both non-HPV and high-risk HPV associated lesions.

5.2 MECHANISMS OF RAPID INDUCTION OF CENTRIOLE MULTIPLICATION BY HPV-16 E7

Elucidation of some of the molecular components necessary for centriole multiplication provided new insights into the mechanism of HPV-16 E7 induction of this pathway [134]. Here, we show that stable HPV-16 E7 expression promotes excess recruitment of PLK4 to maternal centrioles in the form of aberrant PLK4 dots (Chapter 3). Previous results have shown that the aberrant recruitment of endogenous PLK4 was not sufficient to induce centriole multiplication and that a concomitant increase in PLK4 protein level was necessary (Chapter 2; [134]). We found that HPV-16 E7 expression mediates a small but reproducible increase of PLK4 mRNA levels compared to control cells (Chapter 3). This increase was found to be both pRB- and, at least in part, HDAC-dependent. Finally, we show that upregulation of PLK4 mRNA corresponded with the ability of HPV-16 E7 to induce centriole multiplication. Together, these results demonstrate that enhancement of PLK4 mRNA abundance, possibly in an E2F-dependent manner, and aberrant recruitment of excess PLK4 to maternal centrioles may trigger HPV-16 E7-induced centriole multiplication.

In vitro studies have demonstrated that PLK4 overexpression in tissue culture results in an increase in supernumerary centrosomes and when PLK4 is depleted via RNA interference, centriole numbers are reduced with progressive loss of centrioles and the subsequent development of mono-polar spindles [76]. These observations suggest that a strict control of PLK4 transcript and protein levels is necessary to maintain cell viability. Although the threshold level of PLK4 protein which induces centriole multiplication is not known, our own experiments have shown that very small changes in PLK4 protein level induces a small but significant

percentage of cells to exhibit centriole multiplication which may ultimately promote a tolerable level chromosomal instability (Chapter 2; [134]).

CUL1 depletion by RNA interference phenocopies HPV-16 E7-induced centriole multiplication (Chapter 2; [134]). This suggests that HPV-16 E7 expression may not only upregulate PLK4 mRNA but may also interfere with CUL1-mediated proteolytic control at maternal centrioles in order to promote PLK4 protein accumulation. We were unable to detect a change in CUL1 activity as determined by either decreased neddylation or increased CAND1 association by western blot in HPV-16 E7 expressing cells (data not shown), despite the fact that the CAND1 promoter contains E2F-responsive elements. This does not rule out that small differences of CAND1 protein level at the centrosome may occur that were below detectable levels in our assays. Further experiments are necessary to determine if the HPV-16 E7 oncoprotein may either enhance CSN activity or increase CAND1 abundance at the centrosome, promoting CUL1 deneddylation and dampening SCF-ligase activity at maternal centrioles.

One report suggested that HPV-16 E7 requires the protease calpain to degrade pRB [178]. This finding raises the possibility that HPV-16 E7 may interfere with host cellular proteases, also implicated in PLK4 protein stability, which may promote increased PLK4 protein at maternal centrioles.

5.3 DOES PROTEOLYSIS CONTROL OTHER ASPECTS OF CENTRIOLE BIOGENESIS?

The observation that a small but reproducible percentage of HPV-16 E7 expressing cells exhibited elongated centrioles led us to further examine the mechanisms that control centriole elongation.

Primary cilia form in quiescent cells following migration of the maternal centriole to the cell surface to form the basal body. The basal body then elongates to form primary cilia which act as sensory organelles to control several important cellular signaling pathways [147]. The conversion from a maternal centriole to a basal body is not well understood however, previous reports had suggested that knock-down of Cep97 or CP110 promoted the aberrant formation of primary cilia in cycling cells [145]. Furthermore, overexpression of CP110 led to the suppression of cilia formation in quiescent 3T3 cells [145].

We found that prolonged treatment of U-2 OS/centrin-GFP cells with the protesasome inhibitor Z-L_3VS induced abnormally elongated daughter centrioles an important difference in comparison to basal body formation where elongation originates from the maternal centriole. To exploit this observation, we combined our assay system of Z-L_3VS-induced abnormal centriole elongation with an siRNA screen targeting 127 known centrosomal proteins [152] to identify proteins involved in centriole length control (Chapter 4). We were able to discover seven centrosomal proteins that attenuated abnormal centriole elongation when knocked-down (FOP, CAP350, CPAP, hSAS-6, Cep170, ninein, and C-Nap1) and two centrosomal proteins that promoted this process when depleted (Cep97 and CP110). Subsequently we discovered that treatment with either Z-L_3VS or MG132 led to the selective elongation of daughter centrioles.

Several other groups have recently found that overexpression of the centriolar structural protein CPAP resulted in the formation of extra long centrioles [148-150]. These were shown to represent elongated centrioles, and not cilia, based on transmission electron microscopy comparison between these structures and *bona fide* cilia [149, 150]. Subsequently, it was shown that induction of CPAP overexpression led to the elongation of both mother and daughter centrioles [148]. Further, CPAP protein level demonstrated cell-cycle regulation with low protein level in G_1 which increased as cells entered mitosis. Intriguingly, CPAP protein level was found to be controlled by ubiquitin-mediated degradation through interaction with the APC/C during late mitosis [148].

Our results confirm and extend these findings describing the accumulation of several other structural proteins along with CPAP following Z-L_3VS or MG132 treatment. Furthermore, our findings demonstrate that Z-L_3VS-mediated abnormal centriole elongation may be a more complicated process than was suggested by previous studies. CPAP contains an α/β tubulin-binding domain and overexpression by itself may promote the assembly of centriolar microtubules to promote abnormal centriole elongation [148]. In contrast, abnormal centriole elongation in cells treated with either Z-L_3VS or MG132 may require a number of structural proteins besides CPAP to accumulate and hence requires a functional centrosome for the concentration of these components.

HPV-16 E7 expressing cells accumulate PLK1 whose destruction by the APC/C^{Cdh1}-complex plays a role in mitotic exit in human cells [179, 180]. This overexpression of PLK1 may promote a prolonged mitosis and the aberrant accumulation of centrosomal structural proteins, such as CPAP, which are degraded upon mitotic exit and are also substrates of the APC/C^{Cdh1} [148]. This aberrant increase in centrosomal structural proteins may promote a small percentage

of cells to exhibit elongated centrioles. Moreover, HPV-16 E7 physically interacts with γ-tubulin, an important regulator of centriole biogenesis [74, 82]. This interaction may alter γ-tubulin dynamics facilitating the recruitment of components necessary for centriole elongation around the maternal centriole therefore promoting the abnormal elongation of centrioles we observed.

Collectively, our results revealed an unexpected level of complexity in the maintenance of centriole length control and highlighted the critical role of proteolysis in this process.

5.4 A COMPREHENSIVE MODEL OF HPV-16 E7 MEDIATED DISRUPTION OF CENTRIOLE BIOGENESIS

Based on the results presented in this report, we suggest a model of how HPV-16 E7 leads to a rapid induction of centriole multiplication as a cause of genomic instability and ultimately malignant progression (Figure 24).

HPV-16 E7 promotes the formation of an S-phase like milieu through binding and degradation of pRB family members, interaction with histone deacetylases (HDACs) and inactivation of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. This, in turn, upregulates E2F-mediated gene transcription, including cyclin E, and prevents the inactivation of CDKs. Aberrant CDK2/cyclin E activity promotes the aberrant recruitment of PLK4 to the wall of maternal centrioles (Chapter 2; [134]). Endogenous levels of PLK4 are kept to low levels in order to prevent centriole overduplication from occurring through the action of CUL1-based E3 ligase complexes at maternal centrioles.

However, deregulation of E2F-target expression by HPV-16 E7 may also lead to a modest increase in PLK4 mRNA levels (Chapter 3). This increase in PLK4 mRNA, along with

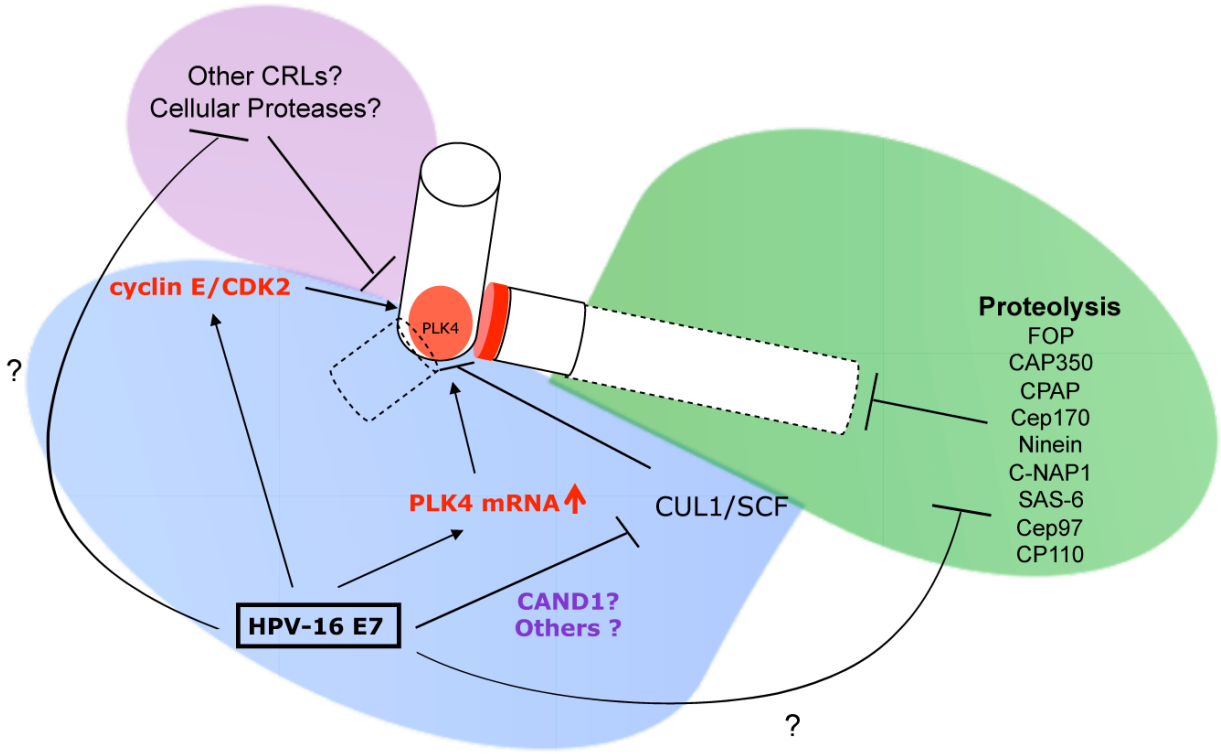

Figure 24. HPV-16 E7 and the complexity of centriole duplication control

HPV-16 E7 induces the formation of an S-phase like milieu through binding and degradation of pRB-family members, interaction with histone deacetylases (HDACs) and inactivation of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, ultimately promoting the deregulation of E2F-mediated gene transcription and the aberrant activation of cyclin E/CDK2 complexes. This promotes centriole multiplication through the aberrant recruitment of PLK4 protein to maternal centrioles in the form of multiple PLK4 dots. A modest increase in PLK4 mRNA, coupled with local interference of SCF-mediated proteolytic control at maternal centrioles, may promote HPV-16 E7 mediated PLK4 protein accumulation. Alternative CRL complexes formed by the remaining six known human cullin-subunits may also play a role in centriole duplication control. A second proteolytic system restrains abnormal daughter centriole elongation and seven centrosomal proteins were found to be necessary for promotion of Z-L_3VS mediated daughter centriole elongation. These results highlight the importance of the proteolytic control of centriole biogenesis and suggest a mechanism by which the HPV-16 E7 oncoprotein promotes centrioles multiplication and ultimately viral carcinogenesis.

de-regulated cyclin E/CDK2, may lead to an accumulation of PLK4 protein at maternal centrioles in the form of aberrant PLK4 dots and ultimately centriole multiplication. An alternate, however not mutually exclusive mechanism, may be the local interfere of CUL1-based E3 ligase control of PLK4 at maternal centrioles by HPV-16 E7 or deregulation of cellular proteases. Disruption of CUL1-based E3 ligase control may occur through the enhancement of CAND1 protein level at the centrosome which may promote PLK4 protein accumulation above a certain threshold level necessary for centriole multiplication. CRL complexes formed by the remaining six known human cullin-subunits may also play a role in centriole duplication control.

HPV-16 E7 was also observed to induce a small but reproducible percentage of cells to exhibit elongated centrioles and we discovered that centriole elongation control also involves proteolysis. These results provide a framework for further studies to explore the regulatory network which controls this process. The biological significance of abnormally elongated centrioles in HPV-16 E7 expressing cells remains to be determined.

Collectively, results presented here highlight the complexity of centriole biogenesis in human cells and underscore the intrinsic ability of HPV-16 E7 to deregulate this process.

5.5 FUTURE DIRECTIONS

Work described in this thesis outlines the role of proteolysis in restraining centriole duplication and how the HPV-16 E7 oncoprotein manipulates this system to induce centriole multiplication. Additionally, we define another role for proteolysis in centriole biogenesis, specifically in the regulation of daughter centriole elongation. Our future work will focus on the following questions: (1) What is the exact role of pRB and HDACs in HPV-16 E7 mediated

upregulation of PLK4 mRNA? (2) Does HPV-16 E7 modulate cellular proteases to induce centriole duplication defects? (3) What is the F-box(es) responsible for PLK4 recognition and subsequent degradation? (4) Can CDK2 and PLK4 small molecule inhibitors be used as therapeutics to prevent the progression of both high-risk HPV and non-HPV associated pre-invasive lesions?

Centrosome abnormalities are found in several human malignancies and have been proposed to be the driving force in promoting chromosomal instability and carcinogenic progression [65, 181]. Based on the findings here, and those reported by others, we believe that CDK2 and PLK4 small molecule inhibitors could be utilized to prevent the progression of pre-invasive high-risk HPV-associated and non-HPV associated lesions [182]. Future studies in our lab will focus on preliminary experiments to determine the efficacy of the combination of these therapies on the prevention of centrosome-mediated genomic instability.

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7.0 DEDICATION

I would like to dedicate this thesis to both my parents and all the wonderful friends I have made on this long journey called graduate school. I am thankful everyday for being lucky enough to have the parents that I do. They taught me to be strong-willed to stand-up for myself and not to let anything get in my way. I know that without their unwavering love and support for me I would not be succeeding as I am now, thank you.

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