# Upstream and downstream of PICH: Revisiting the role of the Plk1-binding protein PICH in the spindle assembly checkpoint


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Hiermit erkläre ich, Nadja Hübner, die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben. Sämtliche Experimente wurden von mir selbst durchgeführt, soweit nicht explizit auf Dritte verwiesen wird. Ich habe weder anderweitig versucht, eine Dissertation einzureichen oder eine Doktorprüfung durchzuführen, noch habe ich diese Dissertation oder Teile derselben einer anderen Prüfungskommission vorgelegt.

München, 30. Juli 2010

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# I. SUMMARY

During mitosis, the flawless distribution of genetic information to two daughter cells is fundamental to the formation and survival of organisms. In eukaryotic cells, the spindle assembly checkpoint (SAC) is the major signaling pathway restraining anaphase onset and mitotic exit until all chromosomes are correctly attached to spindle microtubules via their kinetochores (KTs). A number of evolutionarily conserved proteins, such as Mad2 and its binding partner Mad1, play a key role in SAC signaling. Additionally, protein kinases like Aurora B and Plk1 have been shown to control the timing and correct order of mitotic events through phosphorylation of and crosstalk with SAC components. However, the biochemical mechanism by which these proteins cooperate to regulate the SAC is not yet fully understood.

The DNA-dependent ATPase PICH (Plk1-interacting checkpoint helicase) was identified as a binding partner and substrate of Plk1. PICH shows a very unique localization to KTs and inner centromeres of mitotic chromosomes and ultra-fine DNA bridges during anaphase. Interestingly, PICH spreads over chromosome arms upon depletion or inhibition of Plk1, indicating that PICH localization is controlled by Plk1 kinase activity. Furthermore and most strikingly, depletion of PICH by siRNA abolished the SAC and resulted in an apparently selective loss of Mad2 from KTs, suggesting a role for PICH in the regulation of Mad1-Mad2 interaction.

In the present study, we identified the human Ndc80 complex and the Aurora B kinase, a member of the chromosomal passenger complex (CPC), to be required for PICH localization to KTs and centromeres. We further show that PICH localization to the KTs/centromeres depends on Aurora B, but remarkably not Aurora B kinase activity. In contrast to the Aurora B kinase-independent recruitment of PICH to KTs and centromeres, Aurora B kinase activity seems to be essential for the initial recruitment of PICH to chromosome arms. Moreover, the spindle checkpoint protein Mad2, whose localization normally depends on the CPC, is present at KTs in CPC-depleted cells treated with nocodazole, explaining the mitotic arrest seen under these conditions. Crucially, this result questions a requirement for PICH at KTs for proper Mad2 recruitment.

Reminiscent of the published data on PICH, the protein kinase Tao1 has also been reported as a novel spindle checkpoint component. Intrigued by the proposed function of PICH and Tao1 in SAC signaling, we investigated the molecular mechanism of PICH- and Tao1-mediated Mad2 recruitment to KTs. However, we have subsequently discovered that all PICH- and

Tao1-directed siRNA duplexes that abolish the SAC also reduce Mad2 mRNA and protein expression, contradicting previously published results. Re-expression of murine Mad2 in PICH-depleted cells restored SAC functionality and we identified several siRNA oligonucleotides that effectively deplete PICH or Tao1, without affecting SAC activity or Mad2 localization and expression. In the case of PICH, we discovered that the ability of overexpressed PICH to restore SAC activity in PICH-depleted cells depends on sequestration of Plk1 rather than ATPase activity of PICH. Thus, we argue that the reduction of Plk1 activity (by siRNA-mediated depletion, inhibition or sequestration) partially compensates for reduced Mad2 levels and that Plk1 normally reduces the strength of SAC signaling.

Taken together, the implication of PICH and Tao1 in the spindle checkpoint can be explained by an off-target effect that results in the repression of Mad2 expression. Thus, our results question the role of PICH and Tao1 in SAC functionality and identify Mad2 as a sensitive "off-target" for small RNA duplexes.

# II. INTRODUCTION

## 1. The cell cycle

Cell reproduction is fundamental to the development and function of life. In single-celled organisms, one cell division creates two new organisms. In the development of multi-cellular organisms, countless cell divisions transform a single cell into diverse communities of cells that form the various tissues and organs that comprise the mature creatures. Apart from that, cell division is an essential mechanism to replace dead cells. A series of highly regulated and coordinated events, termed the cell cycle, ensure that a cell duplicates its contents before dividing into two identical daughter cells.

The duplication and division of cellular components must be achieved with extreme precision and reliability in every cycle. This is especially true for the genetic information, encoded in the DNA of chromosomes, which is allowed to duplicate once and only once per cell cycle. In eukaryotic cells, DNA is replicated in S (synthesis) phase, resulting in duplicated chromosomes, called sister chromatids, that then must be equally segregated to the daughter cells during the so-called M (mitotic) phase (Fig. 1).

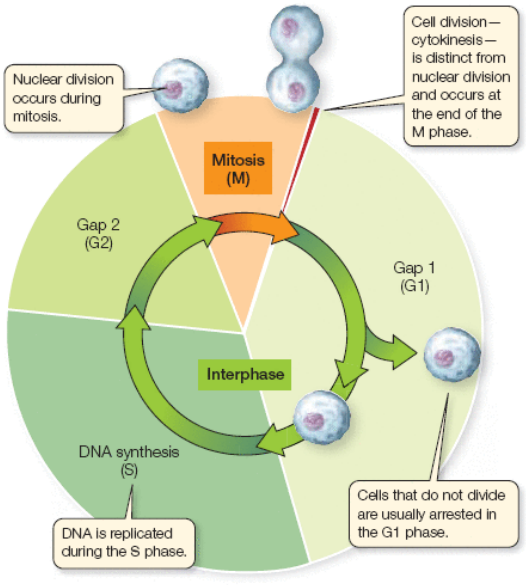



**Figure 1: The eukaryotic cell cycle.** Interphase consists of S, G1 (Gap 1) and G2 (Gap 2) phase. M phase is composed of nuclear division (mitosis) and cell division (cytokinesis). (Adapted from O'Connor, C. (2008) Cell Division: Stages of Mitosis. Nature Education 1(1))

Besides the DNA, a small organelle called centrosome is also duplicated once and only once per cell cycle. The centrosome cycle is tightly coupled to the cell cycle as centrosomes also replicate during S phase and migrate to opposite poles of the cell in the beginning of M phase to organize the mitotic spindle during mitosis. Upon cell division, each daughter cell receives one centrosome. By this, the cell avoids aberrations in centrosome number that have been implicated in chromosomal instability and tumor formation (for review see Nigg, 2007; Nigg and Raff, 2009).

As the integrity of the genome must be maintained, different cell cycle events are highly regulated by various feedback mechanisms, thus ensuring that errors are not propagated. For example, entry into M phase is dependent on DNA synthesis, ensuring that M phase always occurs after S phase. Two gap phases (G1 and G2) respond to both positive and negative growth signals (G1) and prepare the cell for entry into mitosis (G2). An additional phase (G0) refers to a quiescent state in which the cell remains metabolically active, but no longer proliferates unless appropriate extracellular signals are received.

**2. Different stages of mitosis**

In 1882, Walther Flemming was the first cytologist to describe the distribution of chromosomes to daughter cells, a process he called mitosis (Flemming, 1882). The key components of the molecular mechanisms behind Flemming's initial observation were later deduced, resulting in the Nobel Prize in Physiology or Medicine being awarded to Leland H. Hartwell, Tim Hunt and Sir Paul M. Nurse in 2001.

Today we understand mitosis or nuclear division as the part of the cell cycle in which replicated DNA is equally segregated to two daughter cells. Mitosis can be divided into five morphologically distinct phases (Fig. 2). During prophase, chromatin condenses to form chromosomes consisting of two sister chromatids that are tethered together at the centromere, a specialized complex chromatin structure consisting of heterochromatic DNA. The centrosomes, which have also been duplicated during S phase, separate and migrate to opposite poles of the nucleus, thereby allowing their distribution into daughter cells at the end of mitosis. In prometaphase, after nuclear envelope break down (NEBD), specialized structures called kinetochores (KTs) assemble on the centromeric region and are captured by microtubules (MTs), which are nucleated from the centrosomes. This capturing step happens in a highly dynamic and stochastic process. Once the KTs of the sister chromatids are attached to spindle MTs emerging from opposite poles (bipolar attachment) they are moved to the cell equator (metaphase plate) and the cell enters metaphase. After the alignment of all

chromosomes at the metaphase plate, the cell enters anaphase and sister chromatids that have so far been held together by cohesin complexes are pulled apart to opposite poles. Mitosis ends with telophase, during which the mitotic spindle disassembles, sister chromatids decondense and the nuclear envelope reforms. After the formation of daughter nuclei in mitosis, cytoplasmic division (cytokinesis) divides the mother cell into two daughter cells. During this process, the central spindle is compressed into a compact structure called midbody and contraction of an actin-myosin II-based ring-like structure leads to furrow ingression. Finally, abscission takes place resulting in the formation of two genetically identical daughter cells.

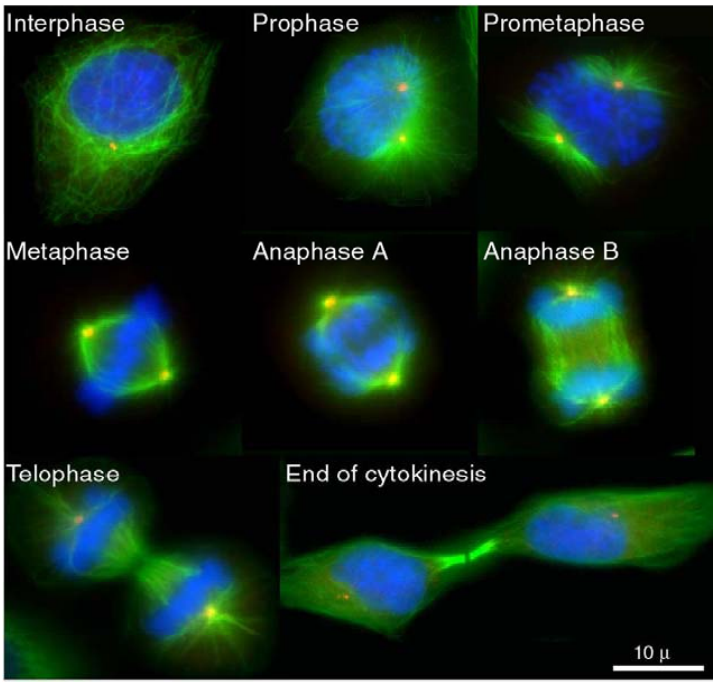



**Figure 2: The different stages of M phase.** The immunofluorescence images illustrate HeLa cells in different cell cycle stages. The mitotic spindle is shown in *green* (α-Tubulin), the centrosomes in *red* (γ-Tubulin) and DNA in *blue* (DAPI staining). (Adapted from Thein, 2007)

### 3. Mitotic kinases

The post-translational modification of proteins by the addition of a phosphate group, a process known as phosphorylation, is mediated by kinases and represents a key regulatory mechanism in many stages of mitosis. This post-translational modification can be removed by protein phosphatases, which allows for meticulous control over tuning of mitotic processes. The key mitotic kinases will be explained in the following section.

### 3.1 Cyclin-dependent kinase 1

The central components of the cell cycle are cyclin-dependent kinases (Cdks). As the cell progresses through the cell cycle, abrupt changes in the enzymatic activities of these kinases lead to changes in the phosphorylation state of other proteins that control the timing of cell cycle events. The enzymatic activities of Cdks oscillate with the levels of so-called cyclins, which bind tightly to Cdks and stimulate their catalytic activity. Furthermore, Cdk activity is controlled by inhibitory phosphorylation events (as discussed below) and inhibitory proteins. Among several Cdk-cyclin complexes, the Cdk1-cyclin B1 complex is the most prominent during mitosis; however, it is clear from genetic studies in yeast that a large network of additional genes also regulates the onset of mitosis. The *Saccharomyces cerevisiae* protein kinase Cdc28p[1] and *Schizosaccharomyces pombe* Cdc2p[2] (later renamed Cdk1) were identified to be regulated by the Cdc25p[3] phosphatase and the Wee1p protein kinase (Nurse and Thuriaux, 1980; Hartwell and Smith, 1985; Russell and Nurse, 1986, 1987). Since then, additional studies have further elucidated the regulation of Cdk1 and its function in mitosis (Doree and Hunt, 2002; Ferrari, 2006). While the activating phosphorylation of Cdk1 seems not to be regulated, the inhibitory phosphorylation events at threonine 14 (Thr14) and tyrosine 15 (Tyr15) by Wee1 and Myt1 (Nigg, 2001) have important functions in Cdk1 regulation (Fig. 3, Krek and Nigg, 1991; Izumi and Maller, 1993). At the G2/M transition Cdk1 enhances its own activity through multiple positive feedback loop mechanisms. For example, Cdk1 stimulates its own activator Cdc25C (Hoffmann et al., 1993) by phosphorylation and simultaneously inactivates its inhibitor Wee1 by phosphorylation followed by subsequent proteasome-dependent degradation (Watanabe et al., 2004; Watanabe et al., 2005). Initially, this and other mitotic events are triggered because the activity of the dual-specificity phosphatase Cdc25C towards Cdk1 exceeds the activity of the opposing kinases Wee1 and Myt1, which function to detect completion of DNA synthesis and successful DNA repair (Ferrari, 2006). Another level of positive Cdk1 regulation is implemented by the stimulation of the Polo-like kinase 1 (Plk1), which further enhances Cdc25C activity by phosphorylation (Fig. 3, Strausfeld et al., 1994; Kumagai and Dunphy, 1996; Barr et al., 2004). Besides stimulation through dephosphorylation, Cdk1 is subjected to an activating phosphorylation in its activation segment or T-loop, which is catalyzed by Cdk-activating kinases (Obaya and Sedivy, 2002).

[1] cell division cycle protein 28
[2] cell division cycle protein 2
[3] cell division cycle protein 25

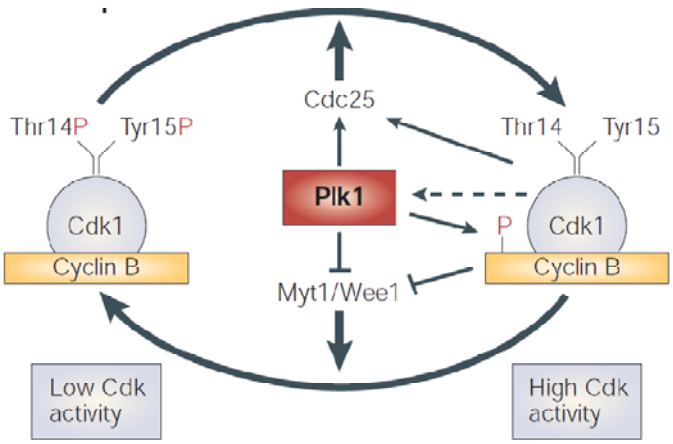



**Figure 3: Cdk1 regulation at the G2/M transition.** B-type cyclins accumulate during G2 phase and the Cdk1-cyclin B1 complex remains inactive by the inhibitory phosphorylations of Cdk1 at Thr14 and Tyr15 by the Wee1 and Myt1 kinases. The Cdk1-cyclin B1 complex is activated when the phosphatase Cdc25 overcomes the opposing effect of Wee1/Myt1. Cdk1 triggers a positive feedback loop by phosphorylating Cdc25 and Wee1/Myt1, thereby causing their further activation and inhibition, respectively. (Adapted from Barr et al., 2004)

The Cdk1-cyclin B1 complex performs numerous different functions during mitosis by phosphorylating several substrates. For example, Cdk1-cyclin B1 is involved in chromosome condensation (Kimura et al., 1998), nuclear envelope breakdown (NEBD) by phosphorylation of lamins (Peter et al., 1991), centrosome separation, and assembly of the mitotic spindle by phosphorylation of MT-associated proteins and the kinesin-related motor protein Eg5 (Blangy et al., 1995). Most importantly, Cdk1-cyclin B1 is involved in the regulation of the anaphase-promoting complex/cyclosome (APC/C, Sudakin et al., 1995; Zachariae et al., 1998). The APC/C is a multisubunit E3 ligase that catalyzes the attachment of ubiquitin chains to mitotic regulators such as cyclin B1, thus promoting their degradation and subsequently inducing mitotic exit (Peters, 2002).

### 3.2 Polo-like kinase 1

In recent years, Polo-like kinases (Plks) have been shown to cooperate with Cdks to regulate cell division. All Plks compromise an N-terminal serine/threonine kinase domain and a highly conserved C-terminal polo-box domain (PBD). The PBD contains two polo boxes that form a binding pocket for phosphorylated motifs in target proteins and thus provides a regulation platform of Plks in time and space. Like many other kinases, Plks are activated by phosphorylation of the T-loop. This mechanism has been postulated to relieve the intramolecular interaction between the kinase domain and the PBD that inhibits the functions of both domains (Mundt et al., 1997; Archambault and Glover, 2009).

The best characterized member of the Plk family, Plk1, has been described from yeast to human and is highly expressed during cell division (Barr et al., 2004). Plk1 has been shown to have multiple mitotic functions and localizes to diverse mitotic structures, including centrosomes, KTs, the central spindle and the midbody (Golsteyn et al., 1995; Lee et al., 1995; Arnaud et al., 1998; Barr et al., 2004). This spatial regulation is mediated by the conserved PBD, which binds to the consensus sequence S-pS/pT-P/X (Leung et al., 2002; Seong et al., 2002; Elia et al., 2003a; Elia et al., 2003b). Hence, only when Plk1 target proteins are phosphorylated at specific sites by appropriate "priming" kinases is the PBD of Plk1 able to dock to its particular target protein (Fig. 4, Lowery et al., 2005).

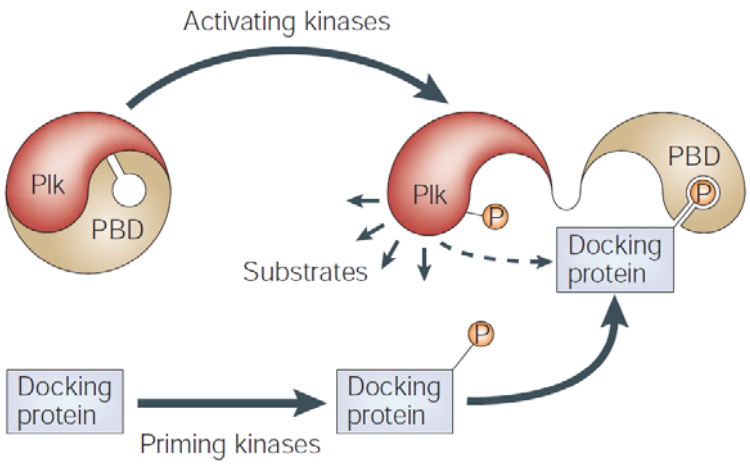



**Figure 4: Schematic illustration of PBD-mediated targeting and Plk1 activation.** Phosphorylation by an upstream kinase on threonine 210 (T-loop activation) stimulates the kinase activity of Plk1. Docking proteins are pre-phosphorylated by priming kinases and targeted to Plk1 in a PBD-dependent manner. (Adapted from Barr et al., 2004)

T-loop activation of Plk1 occurs at threonine 210 (Thr210) and recently Aurora A has been identified to phosphorylate Plk1 at Thr 210 before mitotic entry (Macurek et al., 2008; Seki et al., 2008). The autoinhibitory function of the PBD is further relieved through binding to its "primed" target proteins (Fig. 4, Jang et al., 2002; Hanisch et al., 2006b). Current evidence points to Cdk1-cyclin B1 (Elia et al., 2003b) and MAP[4] kinases (Fabbro et al., 2005) as prominent priming kinases. However, Calmodulin-dependent kinase II (CaMKII, Rauh et al., 2005) as well as Plk1 itself (Neef et al., 2003) have also been shown to function as priming kinases.

[4] mitogen-activated protein

Consistent with the dynamic localization of Plk1, several candidate docking proteins have been described and various mitotic events are linked to Plk1 activity. For example, in early mitosis Plk1 binds to and phosphorylates the centromeric protein INCENP, as well as the checkpoint proteins Bub1[5], BubR1[6] and the DNA-dependent ATPase PICH[7] (Goto et al., 2006; Qi et al., 2006; Baumann et al., 2007; Elowe et al., 2007; Wong and Fang, 2007). During late mitosis, Plk1 targets proteins such as the central spindle proteins Mklp2[8] and PRC1[9] (Carmena et al., 1998; D'Avino et al., 2007).

Plk1 regulates enzymatic activities and cellular functions at many levels. It has been implicated in the activation of Cdk1-cyclin B1 at mitotic entry (Toyoshima-Morimoto et al., 2002; Watanabe et al., 2004), the regulation of centrosome maturation and spindle assembly (Lane and Nigg, 1996; Sumara et al., 2004), the removal of cohesins from chromosome arms (Sumara et al., 2002; Gimenez-Abian et al., 2004), the inactivation of APC/C inhibitors (Rauh et al., 2005), and the regulation of mitotic exit and cytokinesis (Barr et al., 2004; Santamaria et al., 2007).

Thus, Plk1 plays a critical role in the orchestration of cell division, but a complete understanding of Plk1 function during mitosis awaits further investigation (for review see Archambault and Glover, 2009).

### 3.3. Aurora B kinase and the chromosomal passenger complex

The temporal and spatial order of mitotic events is highly coordinated by several proteins and kinases to ensure faithful chromosome segregation. The Aurora family of serine/threonine kinases has been shown to regulate essential processes from mitotic entry to cytokinesis. In mammals, three members of the Aurora kinase family are known: Aurora A, Aurora B and Aurora C. Although Aurora A and B have a very similar amino acid sequence and structure, they have quite distinct localization patterns and functions during mitosis. Probably the regulation of their distinct localization in mitosis explains their different functions.

Aurora A associates with the spindle poles and acts during mitotic entry, centrosome maturation and separation, and the formation of spindle bipolarity (Meraldi et al., 2004). Aurora B is the catalytically active member of the so-called chromosomal passenger complex

[5] Budding uninhibited by benzimidazole 1
[6] Bub-related 1
[7] Plk1-interacting checkpoint helicase
[8] mitotic kinesin-like protein 2
[9] protein regulator of cytokinesis 1

(CPC), a central regulator of mitotic progression. In contrast, little is known about Aurora C but it has been shown to be able to compensate for the loss of Aurora B (Sasai et al., 2004).

The CPC is conserved from yeast to human and consists of the Aurora B kinase, the inner centromere protein (INCENP), the BIR domain containing protein Survivin and Borealin/DasraB. As implicated by its name, the CPC shows a very dynamic localization pattern during mitosis (Fig. 5) and proper localization of the CPC at the right time is essential for faithful execution of mitosis.

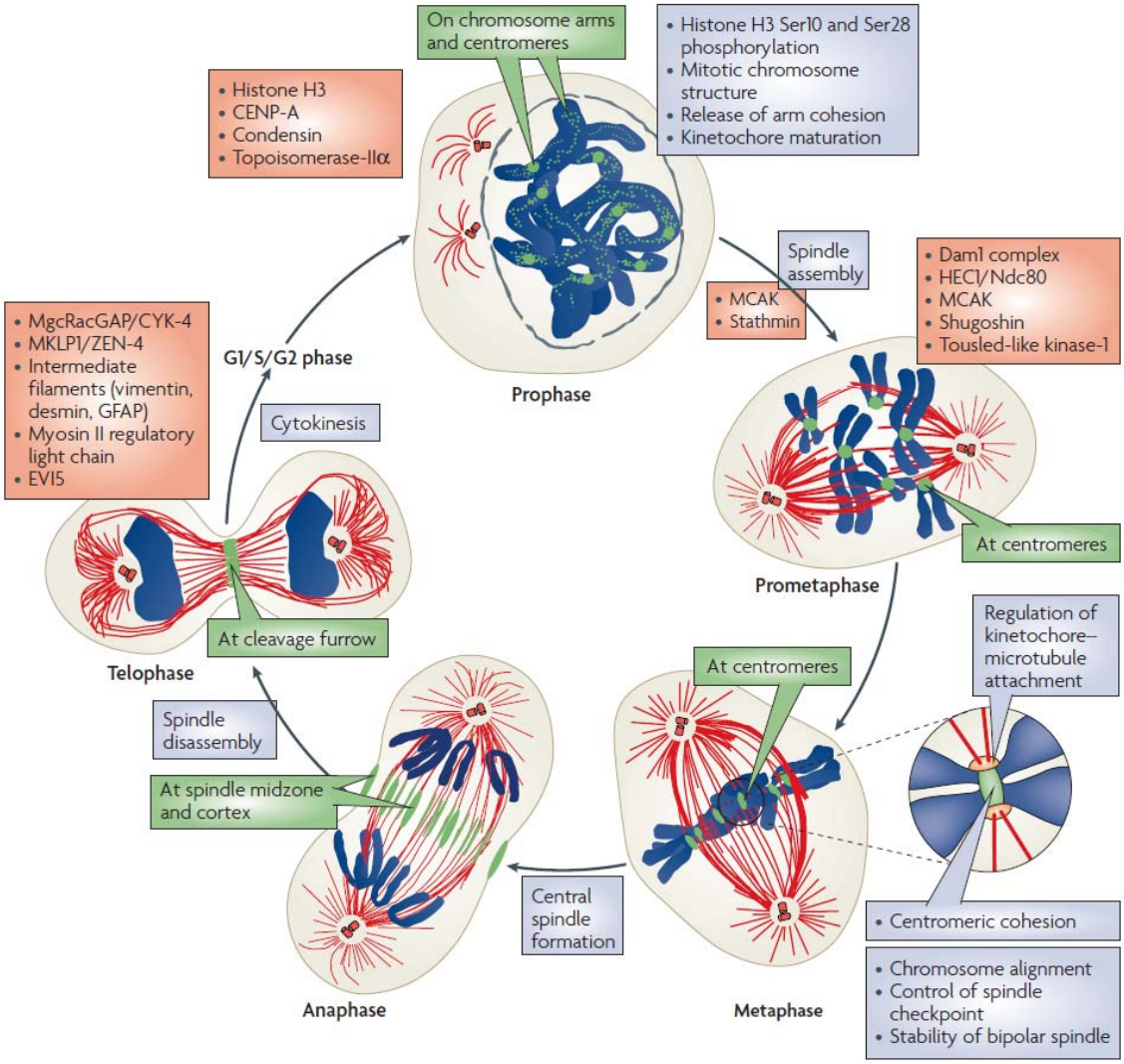



**Figure 5: CPC localization and function during mitosis.** Schematic illustration of CPC localization (*green*), its functions (*blue boxes*) and Aurora B phosphorylation targets (*red boxes*). In prophase, the CPC is involved in the phosphorylation of histone H3, the release of chromosome arm cohesion and the establishment of a functional KT/centromere structure. In metaphase, the CPC localizes to centromeres, where it regulates the KT-MT attachments and SAC activity. Upon the onset of anaphase, the CPC translocates to the spindle midzone where it is involved in the formation of the central spindle. In telophase, the CPC concentrates at the cleavage furrow and subsequently at the midbody, where it regulates cytokinesis. (Adapted from Ruchaud et al., 2007b)

In prophase, the CPC localizes to chromosome arms to control mitotic chromosome structure and dissolution of chromatin arm cohesion. During prometaphase, the CPC is concentrated at the centromere and is involved in the assembly of the KT/centromere region as well as the formation of a stable bipolar spindle. During anaphase, the CPC re-localizes to the central spindle and the cell cortex prior to localizing to the midbody during telophase to ensure proper function of the contractile ring and final abscission (Vagnarelli and Earnshaw, 2004; Vader et al., 2006; Ruchaud et al., 2007b; Vader et al., 2008). Strikingly, members of the CPC are interdependent for localization, meaning that knockdown of any subunit of the CPC leads to loss of the other CPC proteins from the centromere, the central spindle and the midbody. The individual subunits of the CPC will be described in detail in the following section.

INCENP[10] is a 919 amino acid long protein with a coiled-coil region and was the first member of the CPC to be identified (Cooke et al., 1987). As is typical for members of this protein family it has a conserved stretch of 60-80 amino acids at the C-terminal end, called IN-box (Adams et al., 2000). IN-box-mediated INCENP binding to Aurora B increases basal activation of Aurora B, which subsequently leads to INCENP phosphorylation at a conserved TSS motif and autophosphorylation of Aurora B. Through this positive feedback mechanism, Aurora B kinase gains full activity. The N-terminus of INCENP is required for CPC recruitment to the centromere and forms a complex with Survivin and Borealin (Klein et al., 2006). INCENP has also been shown to bind microtubules and to heterochromatin protein-1 (HP1) *in vitro* (Ainsztein et al., 1998; Kang et al., 2001; Wheatley et al., 2001b).

The serine/threonine kinase Aurora B[11] is the catalytic subunit of the CPC and belongs to the Aurora family of kinases (Giet et al., 2005). The kinase activity of Aurora B is directed towards various substrates and requires a ternary complex of INCENP, Borealin and Survivin. In addition to INCENP, Survivin, and Borealin, Aurora B has been demonstrated to phosphorylate proteins at the KT/centromere region, such as CENP-A, Hec1[12] and MCAK[13] (Zeitlin et al., 2001; Andrews et al., 2004; Ohi et al., 2004), at the central spindle and midbody, e.g. the kinesin Mklp1, the GTPase activating protein MgcRacGap, and the intermediate filament vimentin (Goto et al., 2003; Minoshima et al., 2003; Neef et al., 2006). Aurora B phosphorylation is mostly found at the consensus motif $R/KX_{1-3}S/T$ and the protein phosphatase 1 (PP1) has emerged as the major phosphatase opposing Aurora B function

[10] The budding yeast homologue is called Sli15
[11] known as Ipl1 in budding yeast
[12] highly expressed in cancer protein; member of the human Ndc80 complex which further comprises Nuf2, Spc24 and Spc25
[13] mitotic centromere-associated kinesin

(Francisco and Chan, 1994; Emanuele et al., 2008). However, despite the wide body of research, the molecular details of how phosphorylation by Aurora B influences its different substrates are only emerging.

Survivin is a highly conserved member of the inhibitor of apoptosis (IAP) family of anti-apoptotic proteins and is overexpressed in a variety of human cancers (Ambrosini et al., 1997). This family is defined by the presence of a single baculoviral IAP repeat (BIR) $Zn^{2+}$-finger motif. Unlike other IAPs, Survivin lacks a C-terminal RING-finger domain and its role in regulating apoptosis remains under debate. Structural and biochemical studies show that Survivin forms a dimer when present in the cytoplasm (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000). Survivin-specific antibodies however, have identified it as a member of the CPC (Uren et al., 2000; Wheatley et al., 2001a), where it is present as a monomer and its anti-apoptotic function may be abrogated (Ruchaud et al., 2007a). Within the CPC, Survivin appears to be involved in targeting the complex to centromeres (Vader et al., 2006); its role in regulating Aurora B kinase activity remains controversial (Lens and Medema, 2003; Delacour-Larose et al., 2007; Wheatley et al., 2007).

The newest member of the CPC is Borealin/Dasra B which was discovered in 2004 by two independent screens (Gassmann et al., 2004; Sampath et al., 2004). In human, Borealin is suggested to promote local clustering of the CPC that leads to Aurora B autoactivation at the centromere (Sessa et al., 2005; Kelly et al., 2007). Like INCENP and Survivin, Borealin is also phosphorylated by Aurora B *in vitro* (Gassmann et al., 2004). Recently, it has been shown that phosphorylation of Borealin by the checkpoint kinase Mps1[14] leads to increased activation of Aurora B (Jelluma et al., 2008). Except for human, several species including *Xenopus* and chicken express two Borealin isoforms. Paralogues of Borealin, namely Borealin 2/Dasra A, and Australin, seem to be important for the assembly of mitotic spindles in the absence of centrosomes and appear to be meiosis-specific (Sampath et al., 2004; Gao et al., 2008).

The CPC has been implicated in many mitotic functions and siRNA-mediated knockdown of any subunit leads to a delay in prometaphase, lagging chromosomes in anaphase and subsequently results in a cytokinesis defect. Furthermore, Aurora B is required for SAC function in response to lack of tension. In vertebrates, CPC depletion or treatment of cells with the Aurora B kinase inhibitors Hesperadin or ZM447439 (Ditchfield et al., 2003; Hauf et al., 2003; Ditchfield et al., 2005) results in a SAC-dependent arrest when cells are treated with

[14] multipolar spindle 1, also known as dual specificity protein kinase TTK

the spindle poison nocodazole (no KT-MT attachment) but in an override of a taxol-induced mitotic arrest (KT-MT attachment but no establishment of tension). Thus, the CPC has been proposed to sense tension at the KT/centromere region by an unknown mechanism (Cimini, 2007; Musacchio and Salmon, 2007).

Several studies have shown that Aurora B participates in the control of sister chromatid cohesion prior to onset of anaphase (Losada et al., 2002; Gimenez-Abian et al., 2004). In vertebrates, sister chromatids are paired by virtue of a ring-shaped multi-protein complex, called cohesin, which encloses the two DNA double strands. In order to separate sister chromatids during mitosis, cohesin is released in two stages (Waizenegger et al., 2000; Hauf et al., 2005). In early mitosis, Aurora B and Plk1 control the dissolution of cohesion between chromosome arms in the so-called prophase pathway. A small fraction of cohesin at the centromere however, is sufficient to sustain sister chromatid pairing until anaphase onset (Losada et al., 2002; Sumara et al., 2002; Hauf et al., 2005). Centromeric cohesin is protected by the Shugoshin protein Sgo1, whose localization depends on the protein phosphatase 2A (PP2A), Aurora B and Bub1 (Sumara et al., 2002; Kitajima et al., 2005; Kitajima et al., 2006). The final release of sister chromatid cohesion is triggered by separase after spindle checkpoint inactivation and the APC/C-mediated proteasomal degradation of securin. Recently, Aurora B was shown to be involved in regulating the association of separase with mitotic chromosomes (Yuan et al., 2009).

## 4. The kinetochore and centromere region

Each sister chromatid harbors a specialized chromatin region that consists of a series of α-satellite DNA repeats called the centromere. Specialized centromeric components include the histone H3 variants CENP-A, H3K4 and H3K9 (histone H3 methylated on lysine 4 or 9, respectively) and the heterchromatin-protein-1 (HP1) that associates with H3K9 (Palmer et al., 1991; Cleveland et al., 2003; Sullivan and Karpen, 2004), as well as additional *cen*tromeric *p*roteins (CENPs, Foltz et al., 2006). The centromeric region acts as a loading platform on which proteins assemble to build the KT upon entry into mitosis. KTs are specialized protein complexes which are located on opposite sides of the centromeric region and function as major sites of MT attachment (Brinkley and Stubblefield, 1966; Jokelainen, 1967). Although the exact boundaries between centromere and KT are rather loosely defined, electron microscopy analysis revealed that the vertebrate KT is composed of at least three distinct layers (Brinkley and Stubblefield, 1966; McEwen et al., 2007). These are referred to as the centromere, the inner KT and the outer KT (Fig. 6). This tri-laminar structure mediates

the MT-dependent chromosome movement during mitosis. Furthermore, the localization of KT-associated checkpoint proteins is intimately related to the detection of erroneous attachments by the SAC, which delays metaphase to anaphase transition until all chromosomes have properly aligned at the metaphase plate. Thus, it is current challenge to clarify the link between KT-MT binding and SAC signaling (Stukenberg and Foltz, 2010).

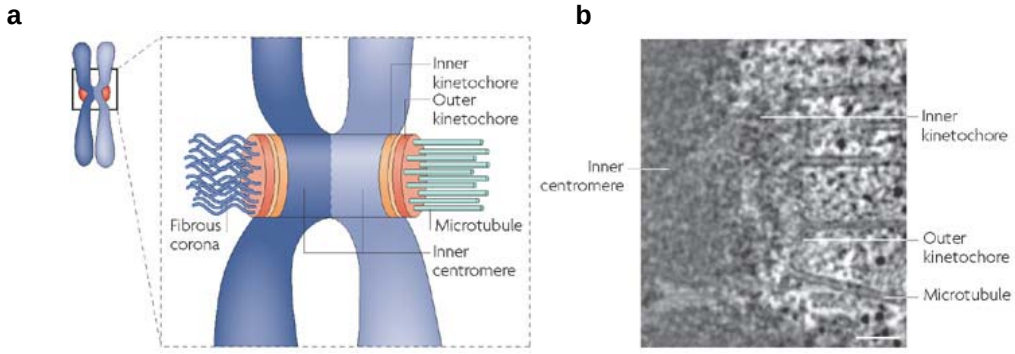



**Figure 6: Molecular architecture of the KT-MT interface. a** Schematic illustration of a mitotic chromosome with paired sister chromatids. The inner centromere, inner KT, the outer KT, and the fibrous corona (left KT) are shown. The right sister chromatid is attached to microtubules. **b** Electron micrograph of a human KT. *Scale bar*, 100 nm. (Adapted from Cheeseman and Desai, 2008)

Besides proteins that localize constitutively to the centromere (see above), a number of components, such as the CPC proteins, proteins involved in sister chromatid cohesion, or the DNA-dependent ATPase PICH associate with the centromere only during mitosis (Maiato et al., 2004; Baumann et al., 2007; Cheeseman and Desai, 2008).

The detailed function of centromeric proteins is still under investigation. However, when pulling MTs stretch centromeres apart during mitosis, a portion of the centromere components eventually extend outwards to the KT (Gorbsky, 2004), regulating the KT-MT interactions and correcting improper MT attachments (Andrews et al., 2004).

The fundamental determinant of KT specification is CENP-A (Howman et al., 2000). CENP-A and other constitutive proteins mark the inner plate of mammalian KTs, which is located at the periphery of centromeric chromatin. It is the earliest protein recruited during KT assembly and is required for the recruitment of other well established KT components. Other molecules that are involved in KT assembly are the members of the CCAN (constitutive centromere associated network) complex. The CCAN comprises proteins co-purifying and localizing with CENP-A. However, the complete function and interdependence of the CCAN proteins is not yet fully understood.

The structural core of the KT comprises a supercomplex of proteins, summarized in the KMN (*K*nl1/Spc105/Blinkin, *M*is12 complex and *N*dc80 complex) network. The Mis12 complex[15] is first to be recruited to centromeres and depletion of the Mis12 complex results in defects in chromosome alignment, orientation, and segregation. Spc105 (also known as Knl1 or Blinkin in humans) appears to serve as scaffold to which other KT components bind and has been shown to interact with the Mis12 complex. Another important complex of the layer is the Ndc80 complex, which provides the molecular basis for the interaction of the KT with MT (Fig. 7, Stukenberg and Foltz, 2010; Vos et al., 2006; Przewloka and Glover, 2009).

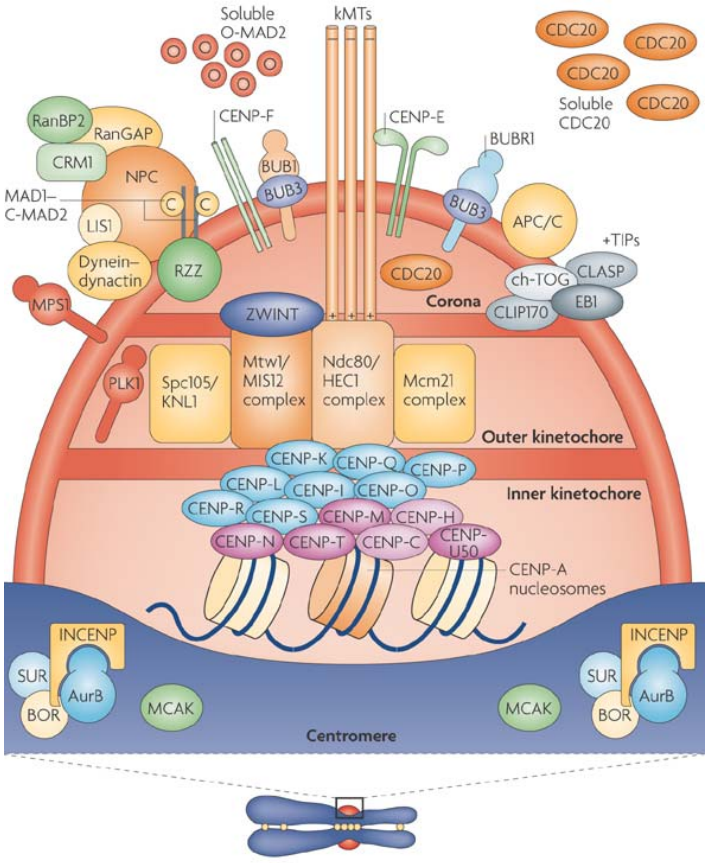



**Figure 7: The kinetochore-centromere region.** Schematic illustration of the spatial distribution of centromere and kinetochore associated proteins. Many proteins, including the Ndc80, Mis12 and Mcm21 (minichromosome maintenance protein-21) complex are recruited to the outer kinetochore specifically in mitosis. Physical interaction of protein-protein complexes is indicated by shapes that contact each other. (Adapted from Musacchio and Salmon, 2007)

The proteinaceous outer KT plate and its associated fibrous corona only form after nuclear envelope breakdown (NEBD). More than 80 proteins have been identified to localize to this layer in human cells (Fig. 7). The RZZ complex for example, comprising *R*ough-deal (Rod),

[15] further comprising the proteins Nnf1, Nsl1, and Dsn1

*Z*este-white 10 (ZW10), and *Z*wilch was identified to associate the proteins of the KMN network and has been implicated in controlling end-on MT attachments (for review see Przewloka and Glover, 2009).

In addition to 20-30 attachment sites for the MT plus-ends, the KT harbors structural components involved in KT-MT attachment (Ciferri et al., 2005; DeLuca et al., 2005), protein kinases like Plk1 and Mps1(Arnaud et al., 1998; Abrieu et al., 2001), MT-dependent motor proteins as CENP-E and dynein (Steuer et al., 1990; Yao et al., 1997) and components of the spindle checkpoint including Mad2[16] and BubR1 (Musacchio and Hardwick, 2002). The fact that spindle checkpoint proteins are recruited through KT proteins supports the idea that the checkpoint signal originates from where spindle MTs attach to the KT and/or where tension between sister chromatids is generated. A more detailed list of centromere and KT associated proteins is reviewed by Maiato et al., 2004 and Vos et al., 2006.

The hierarchic order of KT/centromere assembly is a complex and highly dynamic process. One major pathway is controlled by CENP-A (Regnier et al., 2005) directing the Hec1/Ndc80 complex, CENPs, and BubR1 to the KT. Another pathway depends on the Bub1 kinase that recruits CENP-E and CENP-F, Sgo1, and the checkpoint protein Mad2 and BubR1 to the KT (Boyarchuk et al., 2007). The CPC has also been implicated in KT assembly (Vigneron et al., 2004; Liu et al., 2006). Together, these results indicate that there is significant crosstalk between the different branches and that KT/centromere assembly does not occur in a strictly linear fashion.

## 5. The spindle assembly checkpoint – Tension versus attachment

During cell division, spindle MTs attach to the KT region and align chromosomes within the mitotic spindle before equally segregating the replicated sister chromatids between the two daughter cells. As a requirement for the precise distribution of the genome, chromosomes have to achieve bi-orientation, in which each sister KT is attached to the plus-end of MTs that emanate from opposing spindle poles (bipolar or amphitelic attachment). Erroneous or improper KT-MT attachments can produce lagging chromatids and chromosome missegregation in anaphase (Fig. 8). Cells deploy a highly sensitive surveillance mechanism, known as the spindle assembly checkpoint (SAC) which is capable of delaying anaphase if not every chromosome has achieved bi-orientation. Importantly, laser ablation experiments

[16] mitotic arrest deficient 2

demonstrated that mitotic delay is mediated by an inhibitory signal capable of being generated by only one unattached KT (Rieder et al., 1995).

Among different erroneous KT-MT attachments, monotelic and syntelic attachments can be recognized by the SAC (Fig. 8). Monotelic attachment is characterized by the attachment of only one KT to MTs and represents a normal condition in prometaphase. Syntelic attachments occur when MTs emanating from only one pole might become attached to both sister KTs of a single chromosome. Importantly, the SAC is activated by syntelic attachments as KT-MT interactions are destabilized at low KT tension, which leads to the correction of the misattachment. High tension between amphitelic attached KTs, however, stabilizes the KT-MT interaction (Nicklas et al., 2001; Dewar et al., 2004). In contrast to syntelic attachments, the capture of a single KT by MTs emanating from opposite poles (merotelic attachment) produces sufficient occupancy and tension and thus merotelic attachments are not sensed by the SAC. In any case, destabilization of KT-MT interactions is achieved by the action of Aurora B kinase, the catalytic subunit of the CPC, probably via regulation of the MT depolymerase MCAK (Fig. 8, Biggins and Murray, 2001; Tanaka, 2002; Ditchfield et al., 2003; Hauf et al., 2003; Andrews et al., 2004; Giet et al., 2005).

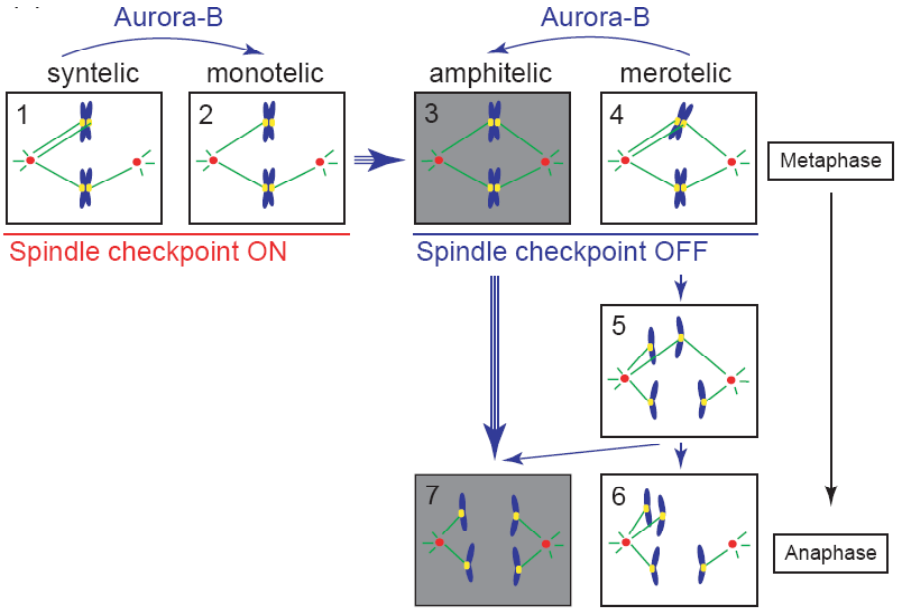



**Figure 8: Schematic illustration of KT-MT attachments.** Amphitelic attachments (3) satisfy the SAC and promote anaphase onset (7). Due to the lack of tensions between sister KTs, the SAC remains "on" when syntelic (1) and monotelic (2) attachments occur and sister chromatid separation cannot proceed. However, the SAC does not sense merotelic attachments (4). Uncorrected attachments will result in chromosome missegreagation during anaphase (5 and 6). Erroneous attachments can be corrected by the action of the Aurora B kinase. (Adapted from Giet et al., 2005)

Whether lack of MT attachment and/or the lack of tension directly produces a "wait-anaphase" signal or whether the SAC indirectly does so by reducing KT-MT attachment through the activity of Aurora B remains to be clarified (Maresca and Salmon, 2010; Pinsky and Biggins, 2005). Recent work in which mechanical changes within the KT itself have been characterized revealed that the "intrakinetochore stretch" has implications for both of these possibilities (Dong et al., 2007; McIntosh et al., 2008).

In 1991, two independent screens in yeast identified various genes, the mutation of which bypassed the ability to induce a mitotic arrest in response to spindle poisons (Hoyt et al., 1991; Li and Murray, 1991). These genes included the MAD (mitotic-arrest deficient) genes Mad1, Mad2 and Mad3 (BubR1 in humans) and BUB (budding uninhibited by benzimidazole) gene Bub1 and were later found to be crucial for the maintenance of the SAC in all eukaryotes. While these so-called checkpoint proteins are highly enriched at unattached KTs delaying anaphase onset, they are absent from properly attached KTs (Chen et al., 1996; Taylor and McKeon, 1997).

The checkpoint protein Mad2 has been identified as one major component of the SAC. Recently, it has been shown that Mad2 adopts two different conformations known as open-Mad2 (O-Mad2) and closed Mad2 (C-Mad2, Mapelli et al., 2007; Musacchio and Salmon, 2007; Luo and Yu, 2008). SAC silencing requires the transition of Mad2 from the inactive O-Mad2 to the active C-Mad2 conformation. This transition probably occurs at the KT and is mediated by the binding of Mad2 to its interaction partner Mad1. In other words, a mobile (O-Mad2) and immobile (Mad1-C-Mad2) fraction of KT-associated Mad2 exists that are essential to turn over O-Mad2 through a mechanism known as conformational dimerization. The generated C-Mad2 targets and inhibits Cdc20, a KT-bound cofactor of the ubiquitin ligase APC/C (Peters, 2006; Yu, 2007). This model therefore is referred to as "template model" as the Mad1-C-Mad2 can be viewed as a template for the formation of a C-Mad2-Cdc20 complex (Musacchio and Salmon, 2007).

Furthermore, a so-called mitotic checkpoint complex (MCC) that contains the SAC proteins Mad2, BubR1 and Bub3, as well as Cdc20 itself, seems to efficiently inhibit the APC/C (Hardwick et al., 2000; Sudakin et al., 2001; Morrow et al., 2005). However, the assembly of the MCC and how the MCC inhibits APC/C activity is poorly understood (Nilsson et al., 2008; Sczaniecka and Hardwick, 2008; Herzog et al., 2009).

As mentioned previously, the SAC negatively regulates the ability of Cdc20 to activate the APC/C-mediated polyubiquitylation of two key substrates, securin and cyclin B, thereby preventing their degradation by the 26S proteasome (Bharadwaj and Yu, 2004; Pines, 2006).

Securin is an inhibitor of separase, a protease that cleaves the cohesin complex which holds sister chromatids together. Cohesin cleavage is required for sister chromatid separation and anaphase onset (Fig. 9). Destruction of cyclin B, on the other hand, inactivates Cdk1, which promotes exit from mitosis (Peters, 2002, 2006).

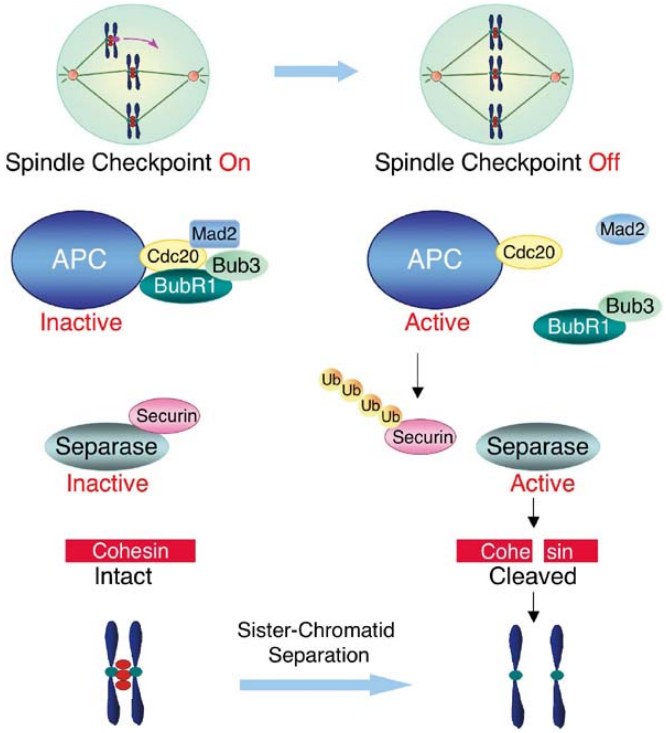



**Figure 9: Simplified view of spindle checkpoint signaling.** In the absence of KT-MT interactions, several activities converge on the creation of the mitotic checkpoint complex (MCC) consisting of Cdc20, BubR1, Bub3 and Mad2, which inhibits the anaphase-promoting complex/cyclosome (APC/C). Although the mechanistic details are still unclear, the Aurora B kinase, Cdk1 and Bub1 might contribute to the formation of the MCC. Bipolar attachment of sister KTs turns the SAC off, activates the APC/C-dependent polyubiquitylation of cyclin B and securin and their subsequent degradation. This in turn allows the execution of anaphase through the separase-mediated cleavage of cohesin. (Adapted from Bharadwaj and Yu, 2004)

Other SAC components include Mad1 and the kinase Bub1, Mps1 and Aurora B which are required to amplify the SAC signal and the rate of MCC formation (Abrieu et al., 2001; Kallio et al., 2002; Hauf et al., 2003; Morrow et al., 2005). How these proteins cooperate to inhibit the APC/C is not yet fully understood, but most models emphasize a key role of Mad2 in Cdc20-APC/C inhibition (Mapelli et al., 2006; Yu, 2006).

Among several mechanisms that have been described to contribute to the inactivation of the SAC after chromosome bi-orientation during mitosis, two are outstanding. First, a key process in tuning off the spindle checkpoint is the “stripping” of Mad1, Mad2, and other checkpoint

proteins from the KTs to the spindle poles by dynein mobility along MTs (Howell et al., 2001; Wojcik et al., 2001; Maiato et al., 2004). In turn, the steady state concentration of SAC proteins at KTs changes and has profound effects on the activity of the spindle checkpoint.
Another mechanism of inactivation is centered on the protein p31$^{comet}$. p31$^{comet}$ binds with high affinity to C-Mad2 thereby competing with O-Mad2 for binding to C-Mad2. Thus, p31$^{comet}$ might function as a brake for the positive feedback loop that is based on C-Mad2 (Musacchio and Salmon, 2007)

### 6. Snf2 type helicases

Several decades ago Gorbalenya and Koonin identified a large group of proteins that share a series of short ordered motifs, today known as helicase motifs (Gorbalenya and Koonin, 1988; Gorbalenya et al., 1988). These motifs were sequentially labeled I, Ia, II, III, IV, V, and VI and include the so-called Walker A and B sequences required for nucleotide binding. Based on similarity, proteins containing these helicase motifs can be subdivided into superfamilies 1, 2 and 3 (SF1, SF2 and SF3, respectively, Subramanya et al., 1996). The Snf2 family constitutes a subclass within the SF2 and contains proteins with a helicase-like region of similar primary sequence to the *Saccaromyces cerevisiae* protein Snf2p. Sequence alignments showed that members of the Snf2 family could be further divided into subfamilies such as Snf2-like, SSO1653-like, Iswi-like and Rad54-like (Fig. 10, Eisen et al., 1995; Flaus et al., 2006).

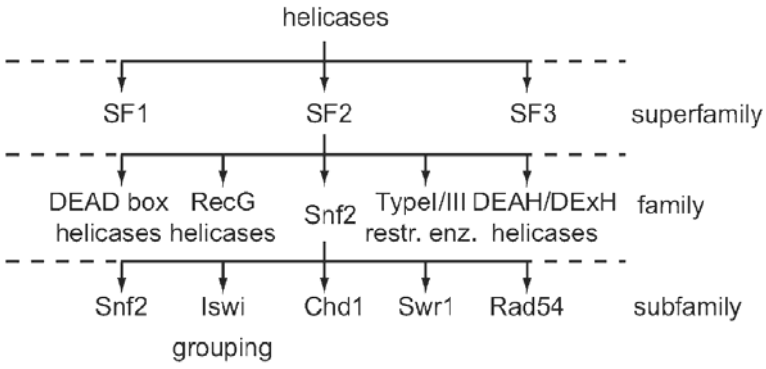



**Figure 10: Schematic tree view of helicases.** The diagram illustrates the hierarchical classification of superfamily (SF), family and subfamily levels. E.g. Rad54 belongs to the Rad54 subfamily of Snf2 type family ATPases, which belong to the helicase superfamily 2 (SF2). (Adapted from Flaus et al., 2006)

The Snf2 protein was initially identified in screens for genes that regulate mating-type switching (SWI) and sucrose non-fermenting (SNF) phenotypes in yeasts (Stern et al., 1984; Nasmyth and Shore, 1987; Neigeborn and Carlson, 1987; Carlson and Laurent, 1994). Later,

Snf2 was found to be the catalytic subunit of the multi-subunit SWI/SNF chromatin-remodeling complex (Becker and Horz, 2002). SWI/SNF proteins are relatively rare in yeast, but 5-7% of all yeast genes require SWI/SNF activity for their expression (Zraly et al., 2006; Monahan et al., 2008). Thus, the SWI/SNF complex does not regulate one exclusive signaling pathway, rather serving as a fundamental component of various essential and often unrelated pathways.

Today, many proteins have been identified that are related to Snf2 through sequence similarity and are often found in larger complexes such as SWI/SNF, RSC[17], NURF[18], ACF[19] and INO80[20]. These complexes contain many polypeptides with a total molecular weight in the megadalton range (Cote et al., 1994; Ito et al., 1997; Shen et al., 2000). Rather than acting as processive helicases, the Snf2 family members generally use ATP hydrolysis to execute a variety of biological processes such as replication, transcription, recombination, translocation along double stranded DNA (dsDNA), DNA repair and chromatin remodeling (Havas et al., 2001; Becker and Horz, 2002; Saha et al., 2002; Svejstrup, 2003; Beerens et al., 2005; Durr et al., 2006; Lia et al., 2006).

In summary, many Snf2 family proteins are part of larger multi-protein complexes and are likely to act to alter chromatin structure. However, detailed insights how ATP-driven changes in duplex DNA is realized are still missing. Future studies will hopefully shed light on the role of the various domains of Snf2 family enzymes and the additional polypeptides and motifs within these complexes.

### 7. PICH – a DNA-dependent ATPase

In 2007, the DNA-dependent ATPase PICH (Plk1-interacting checkpoint "helicase") was found in a Plk1-PBD Far Western ligand binding assay and identified as an interaction partner and substrate of the mitotic kinase Plk1 (Baumann et al., 2007). Database analysis of the domain structure of PICH revealed several characteristics that group PICH into the Snf2 family of helicases. In particular, this protein harbors an N-terminal Snf2-like helicase domain accompanied by a C-terminal HELICc extension, which has high homology to the human Snf2 family members CSB/Ercc6 and Rad54. The GKT sequence (Walker A motif) and the DEXH domain (Walker B motif) are typical for Snf2 helicase domains and required for nucleotide binding and hydrolysis. Thus, it has been suggested that PICH belongs to the

[17] remodel the structure of chromatin
[18] nucleosome remodeling factor
[19] ATP-utilizing chromatin assembly and remodeling factor
[20] inositol requiring 80

subfamilies of Rad54-like helicases or the Snf2 family of helicase superfamily 2 (Eisen et al., 1995; Flaus et al., 2006; Baumann et al., 2007). In addition, a novel motif located C-terminal of the "helicase" domain of PICH which is highly conserved in PICH orthologs has been described as the PICH family domain (PFD, Baumann et al., 2007). Database searches focused on the PFD revealed PICH orthologs in vertebrates and plants as well as in non-vertebrates, in *Dictyostelium* and in the single cell eukaryotic parasite *Entamoeba*, but not in yeast or the typical invertebrate model organisms *Drosophila melanogaster* or *Caenorhabditis elegans*.

PICH localizes to the KT/centromere region of mitotic chromosomes and can be found at ultra-fine DNA bridges or threads containing centromeric DNA (Baumann et al., 2007; Wang et al., 2008). These centromeric DNA-containing threads are progressively stretched and resolved as cells enter anaphase (Wang et al., 2010; Baumann et al., 2007; Wang et al., 2008). PICH localization is altered once Plk1 is inactivated by either siRNA-mediated depletion or inhibition by the small Plk1 inhibitor TAL (Baumann et al., 2007; Santamaria et al., 2007). Interestingly, PICH seems to be required for Plk1 recruitment to chromosome arms (Santamaria et al., 2007) and has been reported to function in chromosome arm cohesion during early mitosis (Leng et al., 2008). Furthermore, it has recently been shown that PICH and co-targeted Plk1 coordinately maintain prometaphase chromosome arm architecture (Kurasawa and Yu-Lee, 2010). In addition to a possible function of PICH at chromosome arms, the KT localization suggests that PICH plays a role in SAC signaling. Strikingly, cells depleted of PICH by siRNA failed to arrest in mitosis when treated with the MT poisons nocodazole or taxol due to SAC failure. This phenotype was further suggested to be due to the inability of PICH-depleted cells to recruit the checkpoint protein Mad2 to KTs (Baumann et al., 2007). Because of its unique localization, its possible interaction with Mad2, and the apparent phenotype to override the SAC, PICH has been proposed to play an essential role in checkpoint signaling, likely as a tension sensor. However, the mechanism of PICH recruitment to the KT/centromere region, its action during checkpoint activation/inactivation and its function at ultra-fine DNA bridges remained elusive (Fig. 11, Baumann et al., 2007).

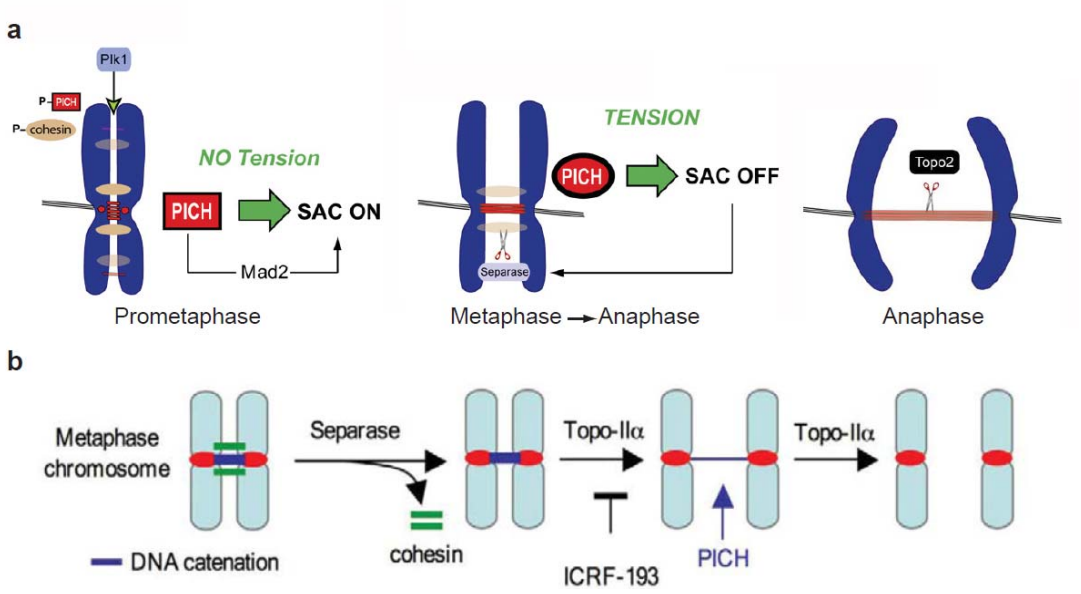



**Figure 11: Working models illustrating the purported function of PICH. a** During prometaphase PICH accumulates at KTs and inner centromeres to uphold conditions permissive for the production of Mad2 protein at the KTs. The SAC is "on" and the APC/C is inhibited. In response to bipolar attachment, the catenated centromeric DNA is stretched under tension, resulting in the formation of PICH positive ultra-fine DNA bridges. A tension-induced change in PICH activity would then bring the production of inhibitory Mad2 to a halt, the SAC is turned "off" and anaphase onset is initiated. PICH remains associated with centromeric DNA threads until decatenation is completed by topoisomerase II. (Adapted from Baumann et al., 2007) **b** Complete metaphase chromosome separation requires the activation of separase, the removal of centromeric cohesion and the topoisomerase II-mediated resolution of centromeric DNA. (Adapted from Wang et al., 2008)

Additional proteins besides PICH have been suggested to play key roles in SAC signaling in recent years. For example, a genome-wide siRNA screen identified a requirement for the Tao1 kinase (also known as microtubule affinity-regulation kinase kinase (MARKK), Timm et al., 2003; Johne et al., 2008) in the SAC (Draviam et al., 2007). Moreover, Tao1 depletion was reported to cause a selective loss of Mad2 but not Mad1 from the KTs (Draviam et al., 2007), highly reminiscent of the data obtained after PICH siRNA (Baumann et al., 2007). However, the detailed mechanism of Tao1-mediated SAC signaling remains unclear and the present work will bring new insights into the originally established role of PICH and Tao1 in spindle checkpoint signaling.

Mitosis was discovered in the early 1880s and subsequent studies provide a sophisticated explanation of the molecular mechanism. However, our understanding of chromatin compaction, spindle dynamics and forces, regulatory mechanisms at a molecular level and the ever growing list of mitotic proteins is still incomplete. Further research is therefore necessary to fully understand the complete and detailed mechanisms involved in mitosis.

# III. AIM OF THIS WORK

PICH, a member of the Snf2 family of proteins and interaction partner and substrate of Plk1 has been proposed to be an essential component of checkpoint signaling. During early mitosis PICH concentrates at the KT/centromere region of mitotic chromosomes. However, PICH can also be found at chromosome arms under conditions of Plk1 depletion or inhibition and contributes to the recruitment of Plk1 to chromosome arms (Baumann et al., 2007; Santamaria et al., 2007). During anaphase, PICH associates with ultra-fine DNA bridges (or PICH-positive treads) that often connect the KTs of separating sister chromatids (Baumann et al., 2007; Wang et al., 2008). The unique localization of PICH to the KT/centromere region, chromosome arms and ultra-fine DNA bridges prompted us to investigate in this study the initial recruitment of PICH to the KT, the centromere and chromosome arms. Specifically we asked, which KT and centromere associated proteins are required to recruit PICH to the KT/centromere and to chromosome arms and which mitotic kinases regulate PICH localization?

Original findings suggested a role for PICH in SAC signaling. Strikingly, siRNA-mediated depletion of PICH caused the selective loss of Mad2, but not the Mad2 binding partner Mad1 from KTs. This mislocalization of Mad2 completely abrogated the SAC and resulted in massive chromosome missegregation (Baumann et al., 2007). In an independent study, the selective loss of Mad2 from KTs was also reported in response to siRNA-mediated depletion of the protein kinase Tao1 (Draviam et al., 2007). Tao1 was suggested to play a key role in regulating the spindle checkpoint, highly reminiscent of the observations for PICH. The similar phenotypes of mitotic cells depleted of PICH and Tao1 prompted us to elucidate the molecular mechanism underlying the purported checkpoint function of these two proteins in more detail in the second part of this work. The published observations suggested that these two proteins might cooperate in regulating the Mad1-Mad2 interaction at KTs. Hence, we wished to combine our knowledge about PICH localization and PICH- and Tao1-mediated Mad2 recruitment to the KT to understand the molecular mechanism behind the action of the proposed spindle checkpoint protein and tension sensor PICH.

# IV. RESULTS

## 1. Regulation of PICH localization to the KT/centromere and chromosome arms

### 1.1. Comparison of PICH localization during mitosis in different cell lines

The DNA-dependent ATPase PICH localizes to the KT/centromere region of mitotic chromosomes from prophase to metaphase and ultra-fine DNA bridges during anaphase in HeLa and U2OS cells (Baumann et al., 2007). Both cell lines are derived from cancer cells, cervical and osteosarcoma, respectively, and carry chromosomal aberrations. To rule out the possibility that the specific staining pattern of PICH is an artificial effect in cancer cell lines, we wished to analyze the localization of PICH in a non transformed and non immortalized cell line in comparison to other cancer cells. The diploid human cell line IMR90, derived from lung embryonic fibroblasts, showed PICH localization to the KT/centromere and PICH-positive threads during mitosis (Fig. 12, *upper panel*), comparable to the observations in HeLa and U2OS cells. IMR90 cells were directly compared to the IMR90E1A cell line, a transformed cell line that expresses the adenoviral oncogene E1A (Fig. 12, *middel panel*).

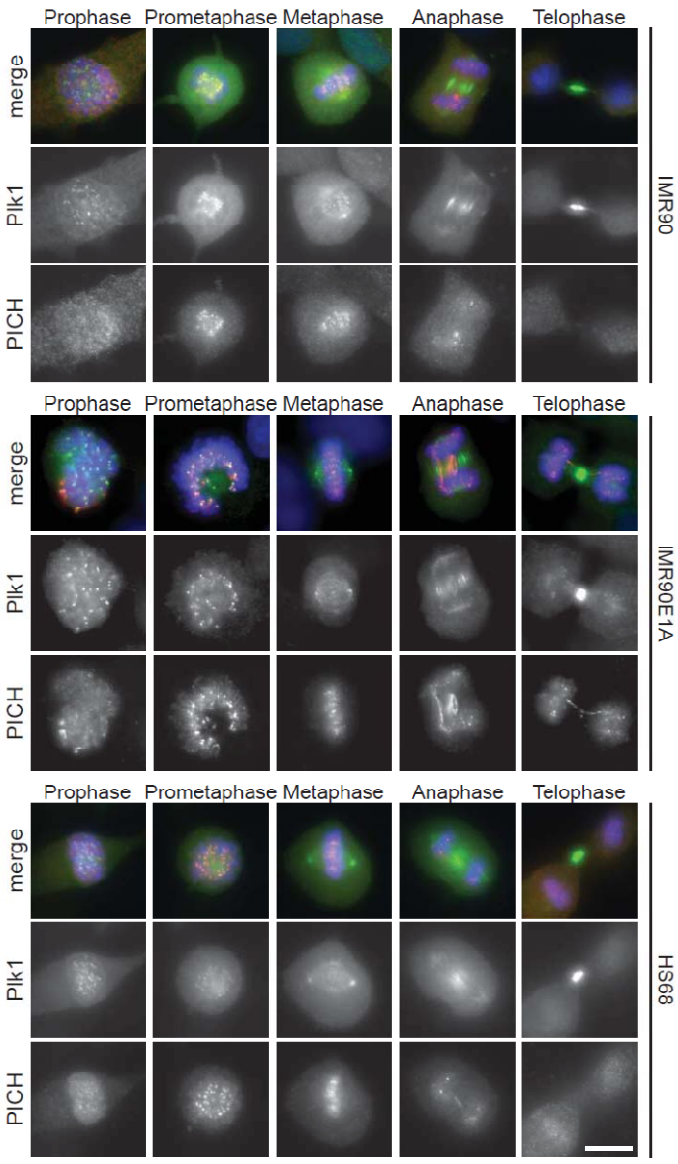



**Figure 12: Localization of PICH in IMR90, IMR90E1A and HS68 cells.** PICH localizes to the KT/centromere region in prometaphase and methaphase and to ultra-fine DNA threads during anaphase in IMR90 (*upper panel*), IMR90E1A (*middle panel*) and HS68 cells (*lower panel*). Plk1 is shown in *green*, PICH in *red* and DNA was stained with DAPI (*blue*). *Scale bar*, 10 µm

E1A proteins are transcription factors that are able to regulate cellular genes such as c-Jun or c-Myc, expression of which leads to the immortalization of cell lines (Houweling et al., 1980). Furthermore, E1A gene products interact with repressors of DNA sequence specific transcription factors such as the retinoblastoma protein pRB. pRB acts as inhibitor of the E2F family of transcription factors, which regulate cell proliferative genes during the cell cycle. As expected from studies in HeLa and U2OS cells, PICH and Plk1 localization appeared normal in IMR90E1A cells and in terminally differentiated human fibroblasts, HS68 cells (Fig. 12, *lower panel*).

### 1.2. Biased screen for proteins that are required for PICH localization to the KT/centromere region of mitotic chromosomes

The correct localization of PICH to the KTs/centromeres of mitotic chromosomes seems to be essential for the precise separation of sister chromatids and mitotic timing (Baumann, 2007). However, little is known about the initial recruitment of PICH to its distinct localizations on mitotic chromosomes. In a systematic survey we searched for upstream components required for the ability of PICH to localize to the KT/centromere. Various candidate KT and centromere-associated proteins were depleted by siRNA and cells were then examined for PICH localization by immunofluorescence. Strikingly, depletion of most of the tested proteins and kinases did not affect PICH localization to the KT/centromere (Fig. 13 and Fig. 14b), with the exception of the CPC and Ndc80 complex (Fig. 14). For example, PICH localization remained unaffected upon depletion of the checkpoint kinases Bub1 and BubR1 as well as the Tao1 kinase that has been implicated in spindle checkpoint signaling (Draviam et al., 2007). As expected, depletion of the motor protein CENP-E resulted in lagging chromosomes and elevated levels of Aurora B; however, PICH levels remained unaltered and are thus not positively regulated by the chromosomal passenger complex (CPC).

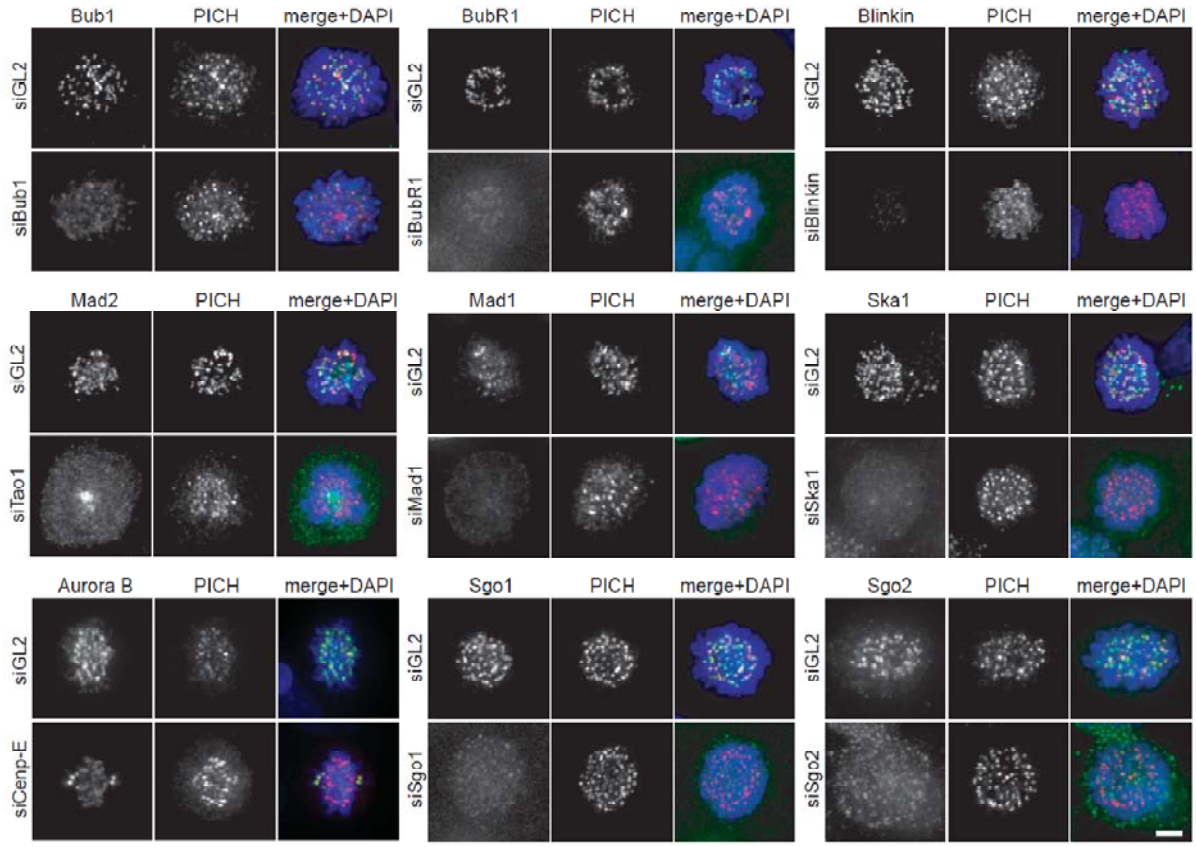
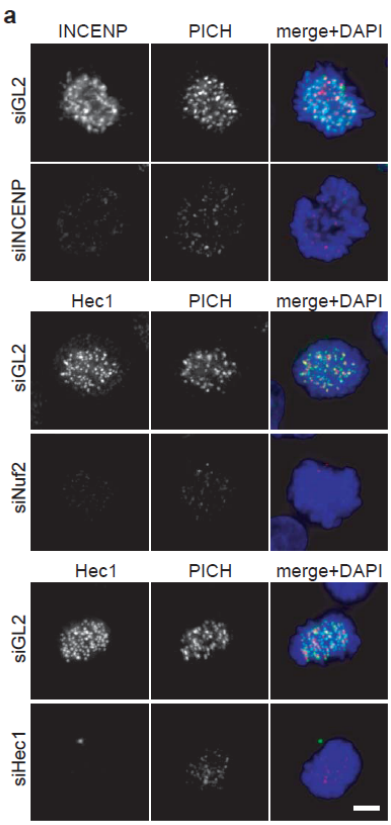



**Figure 13: Focused screen of KT/centromere components that affect PICH localization.** HeLa cells were treated with siRNA duplexes specific for indicated proteins (*green*) for 48 h. Localization of PICH (*red*) to the KT/centromere was analyzed by immunofluorescence. DNA was stained with DAPI (*blue*). Note that depletion of CENP-E is monitored by lagging chromosomes and increased Aurora B staining. Successful Tao1 depletion is monitored by the mislocalization of Mad2. *Scale bar*, 5µm

Most interestingly, siRNA-mediated depletion of the CPC and Ndc80 complex resulted in a significant reduction of PICH signal at KTs and centromeres (Fig. 14a). A summary of all proteins analyzed is shown in Fig. 14b.




b

| siRNA | PICH localization to the centromere/KT |
|---|---|
| Bub1 | + |
| CENP-E | + |
| Sgo2 | + |
| Blinkin | + |
| BubR1 | + |
| Mad1 | + |
| Sgo1 | + |
| Ska1 | + |
| Tao1 | + |
| Aurora B | - |
| Borealin | - |
| INCENP | - |
| Survivin | - |
| Hec1 | - |
| Nuf2 | - |

**Figure 14: Depletion of the CPC and the Ndc80 complex, respectively, mislocalizes PICH from the KT/centromere. a** HeLa cells were depleted of INCENP, Nuf2 or Hec1 and PICH localization was analyzed by immunofluorescence. **b** Summary of focused screen for KT/centromere components that affect PICH localization. + : PICH staining indistinguishable from GL2 siRNA (control). - : PICH staining undetectable at the KTs/centromeres. *Scale bar*, 5 µm.

### 1.3. PICH does not interact with Blinkin

In 2007 the human protein Blinkin (Bub-linking kinetochore protein) was suggested to directly target the checkpoint proteins Bub1 and BubR1 to the KTs (Kiyomitsu et al., 2007). This recruitment is mediated by so-called tetratrichopeptide repeats (TPRs), which are known to facilitate protein-protein interactions (Lamb et al., 1995). Because TRPs have been found at the very N- and C-terminal end of PICH (Baumann et al., 2007) we analyzed a possible interaction between PICH and Blinkin (Fig. 15). Blinkin monoclonal antibodies were used to compare the distribution of Blinkin in mitotic HeLa cells. KT signals were prominent from prophase to anaphase and disappeared in telophase (Fig. 15a). Co-localization at KTs of PICH and Blinkin was detected; however depletion of either protein did not influence the recruitment of the other (Fig. 15b).

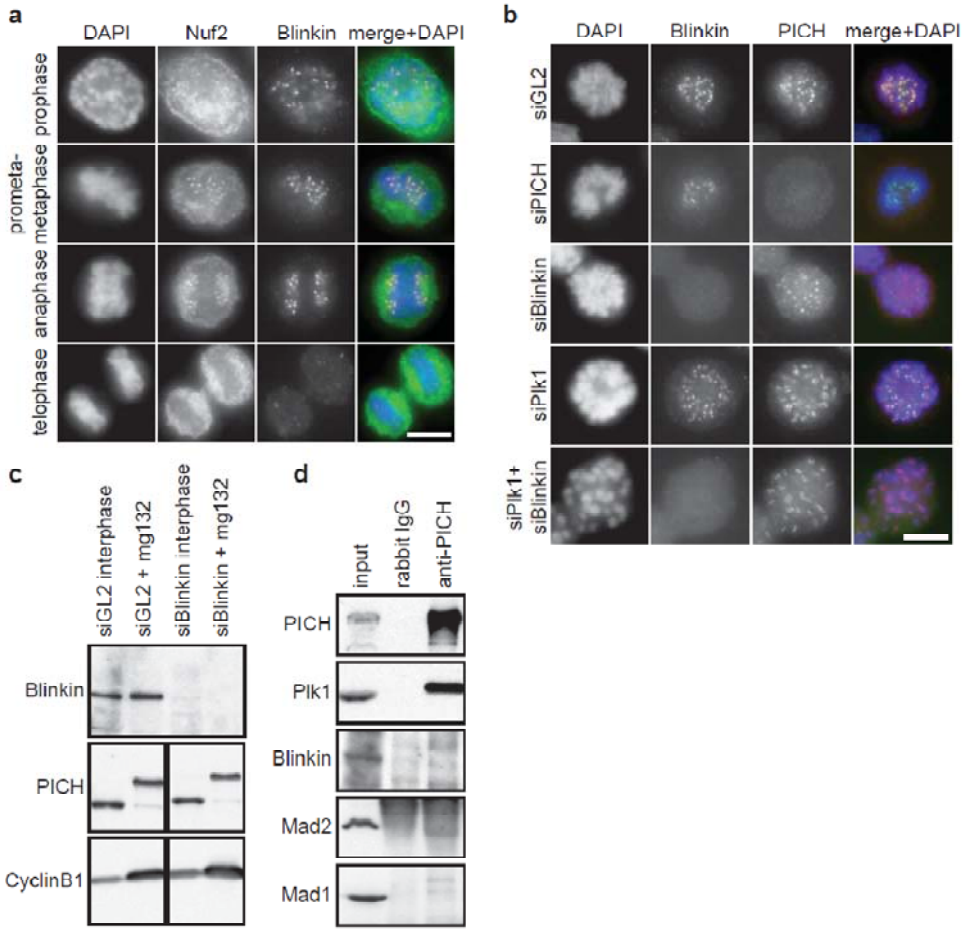



**Figure 15: Blinkin localizes to KTs in mitosis but does not interact with PICH. a** HeLa cells were fixed and stained for Nuf2 (KT localization; *green*) and Blinkin (*red*) in different mitotic stages. *Scale bar*, 10 µm **b** Cells were depleted of indicated proteins by siRNA and stained with antibodies directed against Blinkin (*green*) and PICH (*red*). DNA was stained with DAPI (*blue*). *Scale bar*, 10 µm **c** Depletion of Blinkin from interphase or mitotic cells did not affect PICH protein abundance. Cyclin B1 serves as loading control. **d** Immunoprecipitation with rabbit anti-PICH or rabbit IgG (control) antibodies were performed from nocodazole arrested HeLa cells and probed by Western blotting against the indicated proteins.

In addition we tested the influence of Plk1 depletion on Blinkin localization. While PICH was recruited to chromosome arms in cells depleted of Plk1 (Fig. 15b, Baumann et al., 2007), Blinkin localization remained unaffected. Double depletion of Plk1 and Blinkin resulted in PICH spreading to chromosome arms, indicating that a direct influence of Blinkin on PICH is unlikely. Western blotting indicated that PICH protein abundance is not affected upon depletion of Blinkin by siRNA (Fig. 15c) and *vice versa* (data not shown). To evaluate a possible direct interaction between PICH and Blinkin, PICH was immunoprecipitated from nocodazole arrested HeLa cell lysates and analyzed by Western blotting (Fig. 15d). No interaction was detected between the two candidate proteins under these conditions. These results imply that a direct interaction of PICH and Blinkin is very unlikely.

### 1.4. The CPC and Ndc80 complex act independently in recruiting PICH to KTs and centromeres

The results described in section 1.2. clearly show a requirement for the CPC and the Ndc80 complex for proper PICH localization to the KT/centromere region. Interestingly, they also raise the questions, (1) whether both complexes cooperate in recruiting PICH or act independently of each other and (2) whether direct protein-protein interactions between PICH and the CPC and/or the Ndc80 complex are involved.

In order to understand if these complexes are interdependent or might cooperate in PICH recruitment, both complexes were depleted individually and the dependency for localization on each other was analyzed by immunofluorescence microscopy. Depletion of the CPC by siRNA did not affect Hec1 localization to the KTs, as published previously (Gassmann et al., 2004). Similarly, the CPC was efficiently recruited to the centromere in cells depleted of Hec1 (Fig. 16a). However, PICH was mislocalized in both scenarios as shown before (Fig. 14). This implies that both Aurora B and Hec1 are essential to localize PICH to KTs/centromeres in an independent manner. Western blotting revealed no change in PICH abundance upon depletion or inhibition of Aurora B kinase or depletion of Hec1, respectively (Fig. 16b).

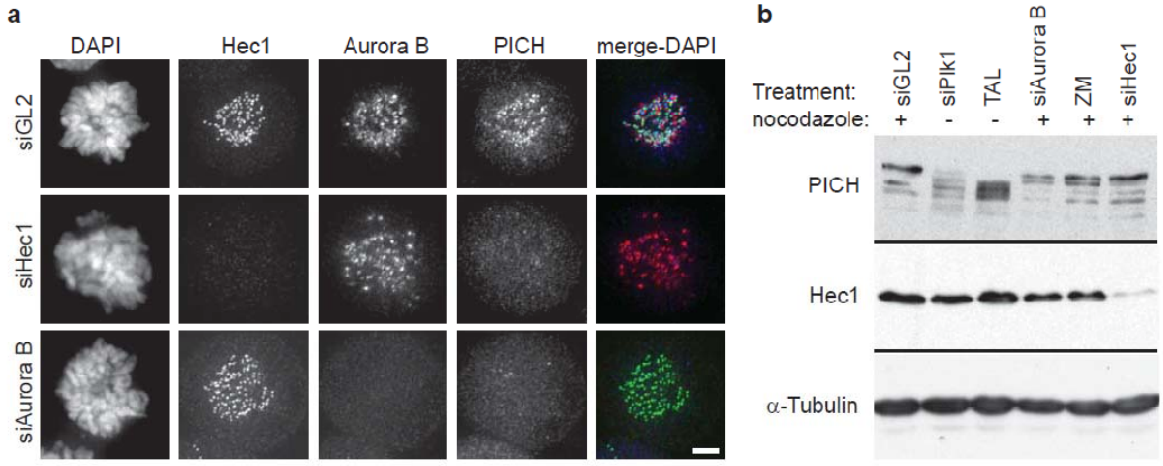



**Figure 16: KT/centromere localization of the CPC and the Ndc80 complex are independent of each other.** **a** HeLa cells were incubated with siRNA duplexes specific for Hec1, Aurora B or GL2 (control) for 48 h. Cells were fixed and subjected to immunofluorescence microscopy with indicated antibodies. *Scale bar*, 5 µm. **b** HeLa cells were depleted of indicated proteins by siRNA for 48 h and treated with nocodazole for 12 h as indicated. Mitotic cells were harvested by shake-off and Western blotting was performed with indicated antibodies. α-Tubulin serves as loading control.

Because PICH has been identified as interaction partner and substrate of Plk1, samples of cell lysates where Plk1 was depleted or inhibited were added as controls. As expected, depletion or inhibition of Plk1 resulted in a significant downshift of PICH (Baumann et al., 2007). In summary, these results confirm that PICH was selectively lost from KTs/centromeres upon depletion of Aurora B or Hec1, respectively, even though the abundance of PICH was not affected.

To test for a direct interaction between PICH and the CPC or Hec1, respectively, co-immunoprecipitation experiments were performed. Specifically, antibodies directed against PICH or Aurora B were used to immunoprecipitate the respective proteins and possible interaction partners were analyzed by Western blotting (Fig. 17). Notably, PICH did not interact with the catalytic subunit of the CPC Aurora B or Hec1 in the conditions tested. Furthermore, Hec1 did not immunoprecipitate with Aurora B, strengthening their independence in complex formation and localization to the KT/centromere.

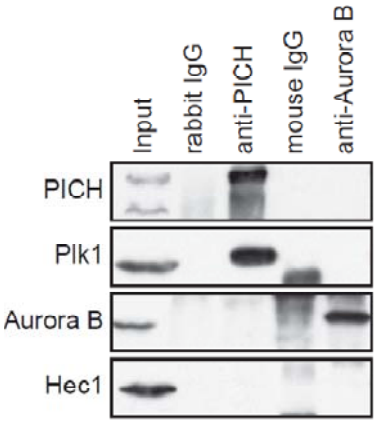



**Figure 17: Co-immunoprecipitation of PICH and Aurora B.** Nocodazole-arrested HeLa cells were collected by mitotic shake off. The cell lysate was incubated with antibody against PICH or Aurora B, respectively, coupled to protein G beads before analysis by Western blotting with indicated antibodies. Mouse and rabbit IgGs were used as control.

The Ndc80 complex, comprising the kinetochore proteins Hec1 and Nuf2 has been shown to be essential for the attachment of spindle MT to the KT (DeLuca et al., 2003; DeLuca et al., 2005). Thus, we investigated if the observed loss of PICH from KTs and centromeres in cells depleted of Hec1 or Nuf2 might be attributed to a general failure in MT-KT interactions. To this end, cells were treated with MT poisons such as nocodazole[21], taxol[22] and noscapine[23] and compared to cells that had been treated with drugs that do not affect MT polymerization or depolymerization such as monastrol (inhibition of the kinesin-related motor protein Eg5, Mayer et al., 1999) or the proteasome inhibitor MG132. DMSO was used as internal control.

[21] nocodazole interferes with the polymerization of microtubules
[22] interferes with the breakdown of microtubules
[23] noscapine appears to interfere with microtubule function

As shown in Fig. 18 treatment with DMSO, MG132 or monastrol did not have any effect on the staining pattern of PICH by immunofluorescence (Fig. 18a-c). The addition of MT poisons however, resulted in an increased of PICH staining in the cytoplasm (Fig. 18d-f), although a significant amount of PICH, detected by the antibody, remained at KTs/centromeres. Thus, loss of PICH from the KT/centromere in Hec1- or Nuf2-depleted cells is unlikely to solely depend on the disruption of MT-KT interaction. However, the possibility remains that intact MTs contribute to PICH recruitment to the KT/centromere.

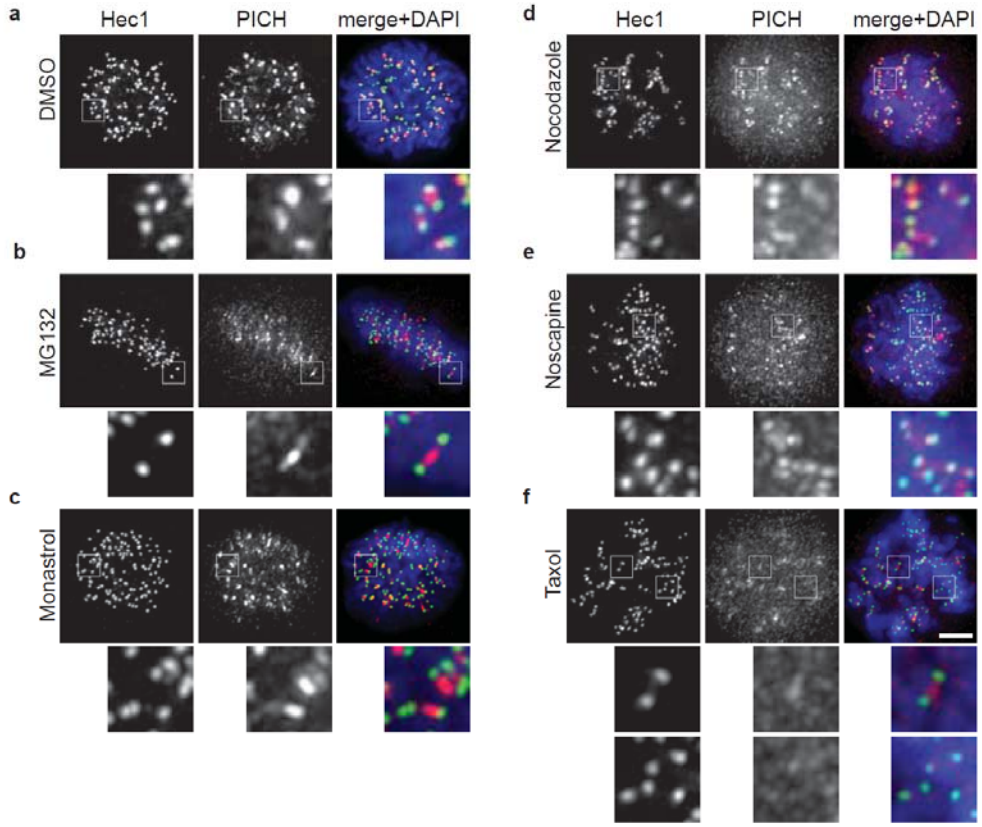



**Figure 18: Microtubule poisons affect but do not abolish PICH localization to the KT/centromere region.** HeLa cells were treated with **a** DMSO (control), **b** MG132 for 2 h, **c** monastrol for 10 h, **d** nocodazole for 10 h, **e** noscapine for 10 h and **f** taxol for 10 h. Hec1 (*green*) and PICH (*red*) localization to the KT/centromere was analyzed by immunofluorescence microscopy. DNA was stained with DAPI (*blue*). *Boxes* indicate regions of blown up images. *Scale bar*, 5 µm

### 1.5. PICH recruitment to the KT/centromere is independent of Aurora B kinase activity

The functions of the CPC in regulating mitotic progression have so far been assigned exclusively to the activity of the Aurora B kinase. Remarkably, cells depleted of chromosomal passenger proteins show phenotypes identical to cells that have been treated with the Aurora B inhibitor ZM447439 (Ditchfield et al., 2003; Hauf et al., 2003). To investigate if the requirement of the CPC in localizing PICH reflects a need for Aurora B kinase activity,

siRNA-mediated depletion of Aurora B was compared to Aurora B kinase inhibition by using small molecule inhibitors. Diminished Aurora B kinase activity was monitored by low levels of phosphorylated CENP-A at serine 7 (S7), an endogenous Aurora B substrate (Fig. 19a, Zeitlin et al., 2001). Interestingly, we found Plk1 to be absent from the KTs under conditions of Aurora B kinase inhibition by ZM447439 (Fig. 19b, see also Klein, 2008) and thus used Plk1 localization as a readout for ZM447439 treatment (Fig. 18b). Furthermore, the finding that Plk1 localization was dependent on active Aurora B kinase is in apparent contradiction to the idea that the phosphorylation status of INCENP by Cdk1 regulates Plk1 localization to the KTs (Goto et al., 2006).

In contrast to Plk1 and most surprisingly, PICH localized to KTs and centromeres when Aurora B kinase activity was inhibited (Fig. 19b). As PICH is found on chromosome arms in cells depleted of Plk1 (Baumann et al., 2007) these observations further imply that cytoplasmic Plk1, as is persists after Aurora B kinase inhibition, is sufficient to remove PICH from chromosome arms. Additionally, it has been shown that Plk1 is mislocalized from KTs upon siRNA-mediated Sgo1 depletion (Pouwels et al., 2007). As shown in Fig. 13, PICH does not spread over chromosome arms after Plk1 mislocalization caused by the depletion of Sgo1, underlining the idea of a catalytically active cytoplasmic pool of Plk1 that functions to remove PICH from chromosome arms during early mitosis.

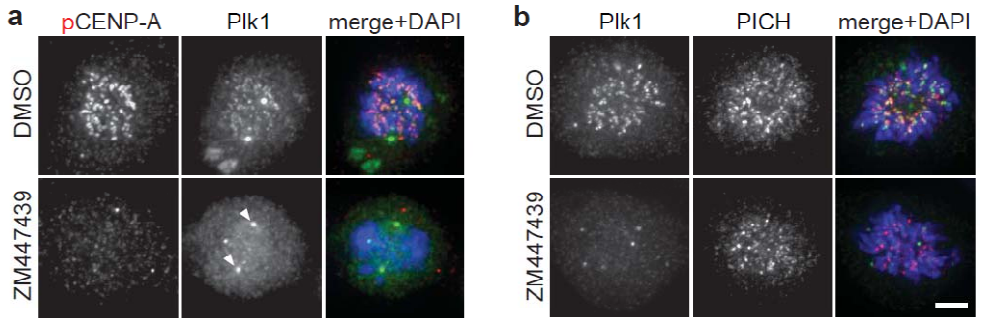



**Figure 19: Aurora B kinase activity regulates Plk1 but not PICH at the KT/centromere. a** HeLa cells were treated with ZM447439 (or DMSO as control) for 2 h, fixed and stained with indicated antibodies. Phospho-S7-CENP-A (pCENP-A) staining (*red*) controls for Aurora B kinase activity. Plk1 (*green*) is absent from KTs but present at the centrosomes under condition of Aurora B kinase inhibition (indicated by *arrows*). **b** In ZM447439 treated cells Plk1 (*green*) is absent from KTs (but present at the centrosomes) while PICH (*red*) localization is unaffected. DNA is shown in *blue* (DAPI). *Scale bar*, 5 µm

To investigate if the observed result was cell line specific, the experiment was repeated in U2OS cells. In agreement with the results obtained in HeLa cells, U2OS cells depleted of

Aurora B by siRNA showed mislocalization of PICH but ZM447439 treatment did not affect PICH localization to KTs/centromeres (Fig. 20, in collaboration with Ulf R. Klein).

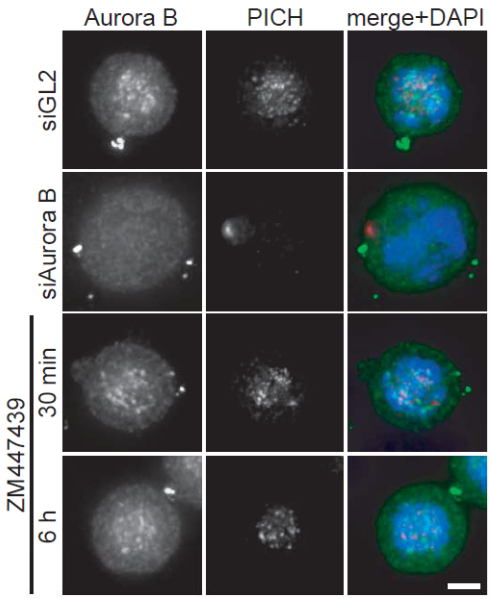



**Figure 20: Aurora B depletion but not inhibition influences PICH in U2OS cells.** U2OS cells were either treated with siRNA oligonucleotides targeting Aurora B or GL2 (control) for 36 h and compared to cells treated with the Aurora B inhibitor ZM447439 for different time points. Aurora B (*green*) and PICH (*red*) were visualized by immunofluorescence. DNA was stained with DAPI (*blue*). *Scale bar*, 10 µm

To exclude possible erroneous results caused by the treatment of cells with one specific inhibitor, we additionally repeated the experiments with the published Aurora B kinase inhibitor VX-680 (Tyler et al., 2007), as well as the Mps1 kinase inhibitor SP600125, which indirectly leads to the downregulation of Aurora B kinase activity (Fig. 21, Zeitlin et al., 2001; Jelluma et al., 2008).

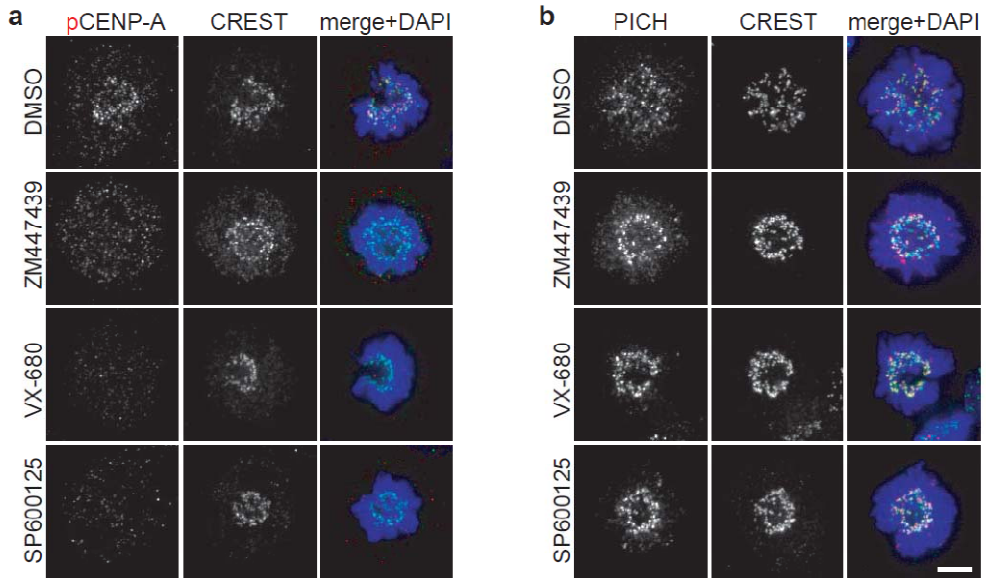



**Figure 21: Comparison of different Aurora B kinase inhibitors.** HeLa cells were treated with the Aurora B kinase inhibitor ZM447439, VX-680, SP600125 and stained for **a** phospho-S7-CENP-A (pCENP-A, *red*) and CREST (*green*) or **b** PICH (*red*) and CREST (*green*), respectively. DNA was stained with DAPI (*blue*). *Scale bar*, 10 µm

After ZM447439, VX-680 and SP600125 treatments pCENP-A staining was clearly diminished, proving Aurora B kinase inhibition (Fig. 21a), while PICH localization was unaffected (Fig. 21b). This result supports the conclusion that PICH recruitment to the KT/centromere is independent of Aurora B kinase activity.

Concerning the localization of PICH, the apparent difference in results observed after ZM447439 treatment and depletion Aurora B by siRNA is rather surprising. Future experiments are required to elucidate possible explanations for this intriguing difference.

### 1.6. Aurora B protein is essential for PICH recruitment to the KT/centromere

Recently, structural data on the CPC has become available, which allows a more detailed view on the interaction sites within the different components of the CPC (Jeyaprakash et al., 2007). To understand which protein of the CPC is essential to recruit PICH to the KT/centromere, siRNA-based complementation assays were performed to analyze two different mutants of the CPC framework protein INCENP. A myc-tagged N-terminal construct of INCENP, comprising amino acids 1-58 ($IN^{1-58}$) and able to bind to Survivin and Borealin but not Aurora B, was available in the lab (Klein et al., 2006). Furthermore, this construct does not comprise the coiled coil region or the IN-box motif of INCENP. However, $IN^{1-58}$ localizes to the centromere of mitotic chromosomes in prometaphase (Klein et al., 2006). A second construct was engineered, in which two point mutations were inserted (F825A and F837A, $IN^{F825A/F837A}$) into myc-tagged full length INCENP. These point mutations abrogated INCENP binding to Aurora B (Sessa et al., 2005), as shown by comparison to myc-tagged wild type INCENP ($IN^{wt}$, kindly provided by Ulf R. Klein, Fig. 22). For a schematic view of constructs used see Fig. 23a.

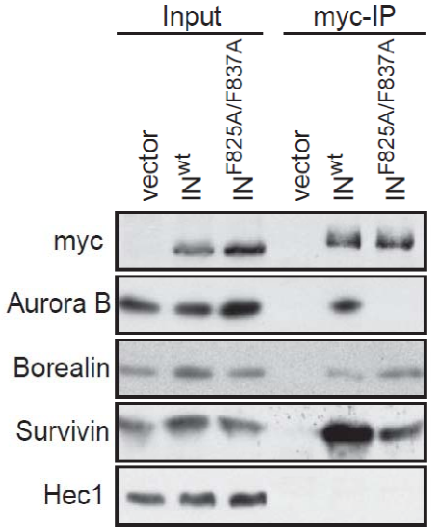



**Figure 22: Construction of an INCENP mutant defective in Aurora B binding.** Indicated myc-tagged INCENP constructs (or the empty myc-vector) were transiently transfected into HeLa cells. Immunoprecipitations with anti-myc specific antibodies were performed from mitotic cell lysates Co-immunoprecipitated proteins were analyzed by Western blotting. Note that all CPC components are present in the complex with wild type INCENP ($IN^{wt}$), however, $IN^{F825A/F837A}$ cannot bind Aurora B but Borealin and Survivin.

To compare the abilities of $IN^{wt}$, $IN^{1-58}$ and $IN^{F825A/F837A}$ to recruit PICH to the KT/centromere, cells were depleted of endogenous INCENP using siRNA oligonucleotides directed against the 3'-UTR and co-transfected with the myc-tagged INCENP constructs. Strikingly, only $IN^{wt}$ was able to restore PICH at KTs, whereas $IN^{1-58}$ and $IN^{F825A/F837A}$ were unable to rescue PICH localization in transfected cells (Fig. 23b). Quantitative analyses are shown in Fig 23c.

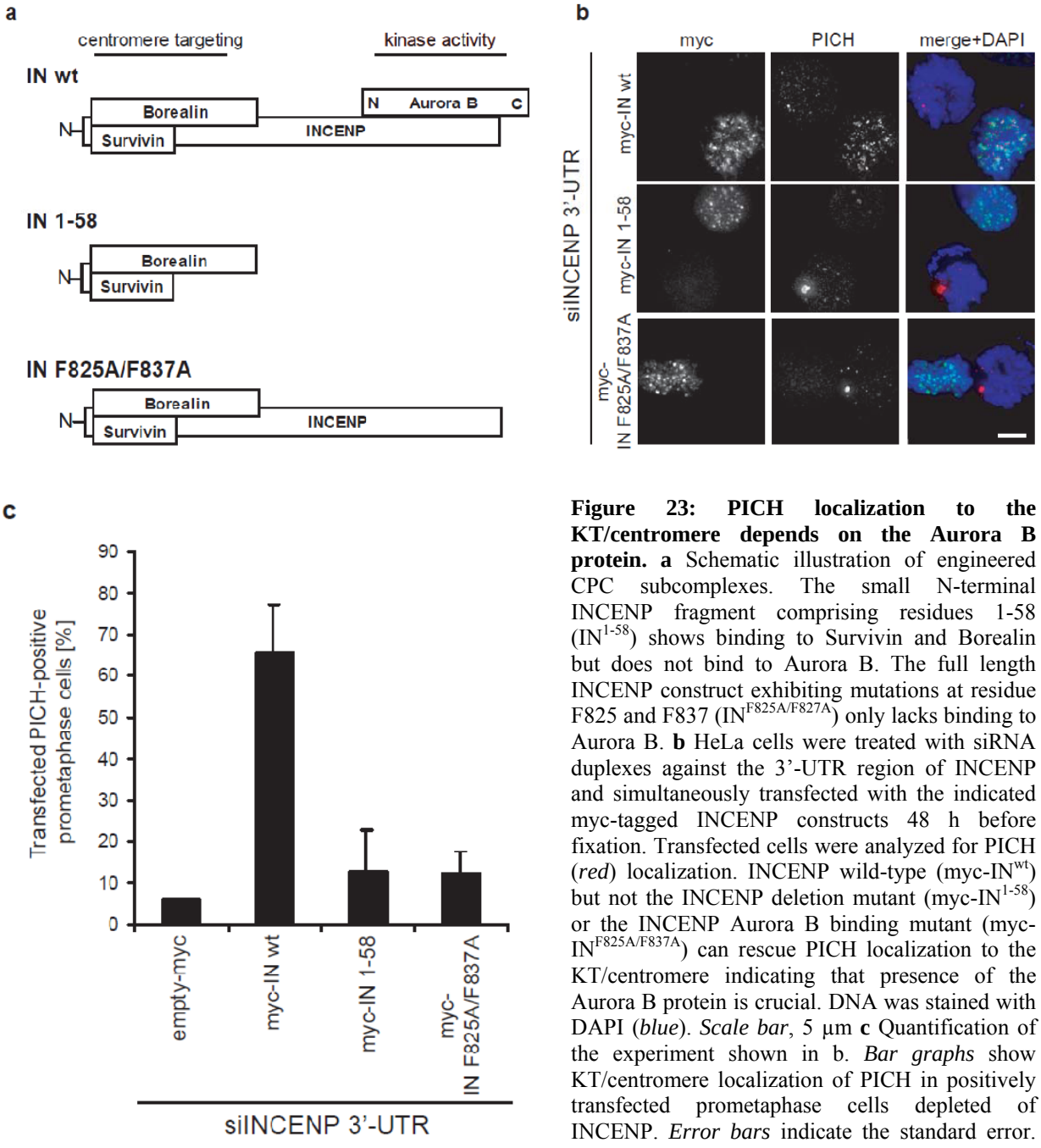



**Figure 23: PICH localization to the KT/centromere depends on the Aurora B protein. a** Schematic illustration of engineered CPC subcomplexes. The small N-terminal INCENP fragment comprising residues 1-58 ($IN^{1-58}$) shows binding to Survivin and Borealin but does not bind to Aurora B. The full length INCENP construct exhibiting mutations at residue F825 and F837 ($IN^{F825A/F827A}$) only lacks binding to Aurora B. **b** HeLa cells were treated with siRNA duplexes against the 3'-UTR region of INCENP and simultaneously transfected with the indicated myc-tagged INCENP constructs 48 h before fixation. Transfected cells were analyzed for PICH (*red*) localization. INCENP wild-type (myc-$IN^{wt}$) but not the INCENP deletion mutant (myc-$IN^{1-58}$) or the INCENP Aurora B binding mutant (myc-$IN^{F825A/F837A}$) can rescue PICH localization to the KT/centromere indicating that presence of the Aurora B protein is crucial. DNA was stained with DAPI (*blue*). *Scale bar*, 5 µm **c** Quantification of the experiment shown in b. *Bar graphs* show KT/centromere localization of PICH in positively transfected prometaphase cells depleted of INCENP. *Error bars* indicate the standard error. Experiment was done in triplicate (n = 18-33).

Because the $IN^{F825A/F837A}$ mutant was not able to restore PICH localization, we exclude the possibility that INCENP, Survivin, and Borealin directly interact with PICH. In contrast, Aurora B protein is essential to target PICH to the KT/centromere. However, co-immunoprecipitation experiments using different protocols and buffer conditions failed to show a direct interaction between Aurora B and PICH (Fig. 17).

### 1.7. Mutual interplay between the CPC, PICH and Plk1 at the KT/centromere

Previous work and this study have shown two components that play key roles in regulating mitosis, namely Aurora B and Plk1, to also regulate PICH localization to the KT/centromere. To test for interdependencies between the CPC, PICH and Plk1 in terms of their KT/centromere localization, HeLa cells were depleted of the respective protein by siRNA treatment and localization of the remaining components was analyzed by immunofluorescence (Fig. 24). PICH depletion did not influence the CPC (using Aurora B staining as readout) or Plk1 localization to the KT/centromere (Fig. 24a+c, Klein, 2008). Knockdown of Plk1 did not affect localization of the CPC but caused re-localization of PICH to chromosome arms, as shown before (Fig. 24b+c, Baumann et al., 2007; Santamaria et al., 2007). Depletion of Aurora B resulted in the mislocalization of PICH, as shown above and also mislocalization of Plk1 (Fig. 24a+b). Western blotting confirmed that the abundance of PICH and Plk1 was not affected by inhibition of Aurora B kinase or depletion of the CPC (Fig. 24d). An antibody directed against phospho-serine 10 histone H3 (pS10H3) was used as readout for Aurora B kinase activity. Taken together, we conclude from these results that Aurora B acts upstream of Plk1 and PICH in a non linear fashion.

Collectively, these data and the results shown in sections 1.5. and 1.6. demonstrate that PICH requires the presence of the Aurora B protein, but not Aurora B kinase activity for its KT/centromere localization. This dependency on CPC integrity but not its enzymatic activity has not been reported for any other KT/centromere associated protein so far.

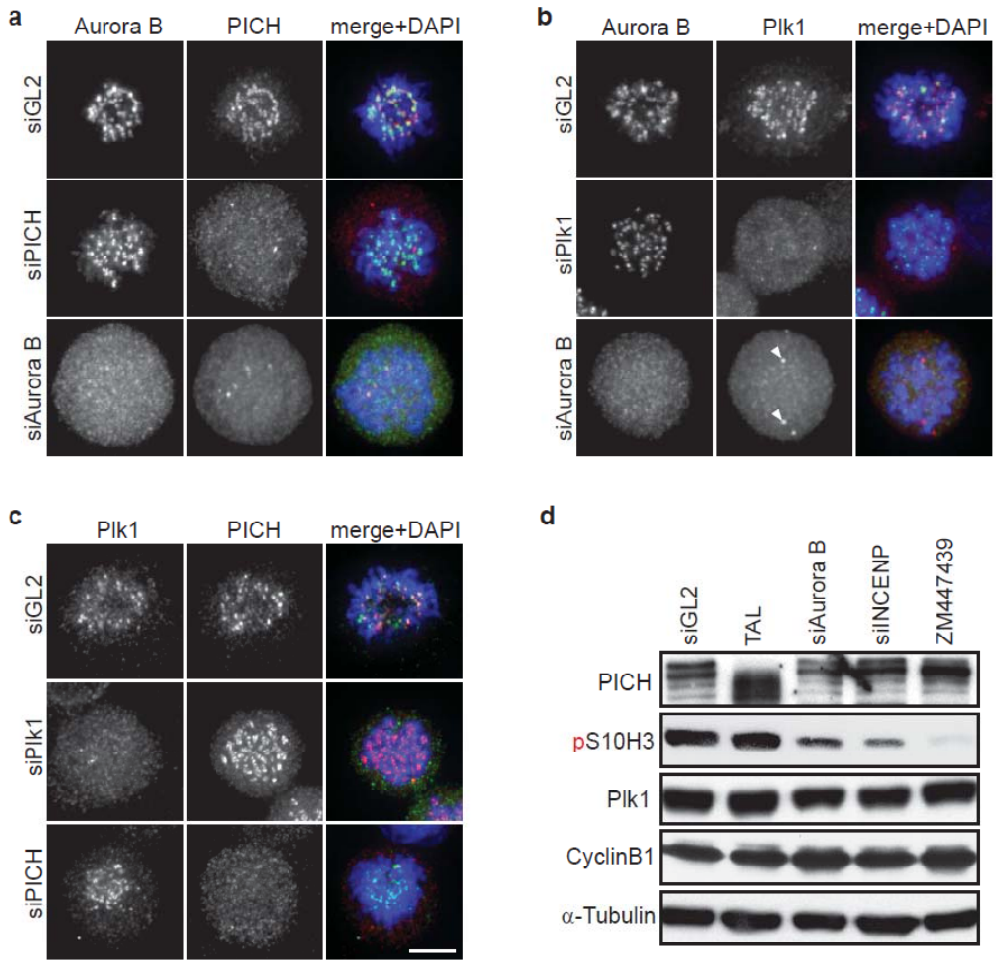



**Figure 24: Interdependence between the CPC, PICH and Plk1 at the KT/centromere.** HeLa cells were treated with siRNA duplexes directed against Aurora B, Plk1 or PICH, respectively, incubated for 48 h and processed for immunofluorescence as indicated. **a** The CPC (*green*) regulates PICH (*red*) but not *vice versa*. **b** The CPC (*green*) regulates Plk1 (*red*) but not *vice versa*. Note that Plk1 is absent from KTs but present at centrosomes after depletion of Aurora B (indicated by *arrows*). **c** Plk1 (*green*) regulates PICH (*red*) but not *vice versa*. DNA was stained with DAPI (*blue*). *Scale bar*, 10 µm **d** HeLa cells were depleted of indicated proteins by siRNA treatment and cell extracts were analyzed by Western blotting. Cyclin B1 and α-Tubulin serve as loading control. pS10H3 indicates the successful inhibition of Aurora B kinase activity.

## 1.8. Regulation of PICH chromosome arm localization

In Plk1-depleted cells PICH localizes to chromosome arms (Baumann et al., 2007). In contrast, siRNA-mediated knockdown of Aurora B led to the mislocalization of Plk1 and the absence of PICH from the KT/centromere. Therefore, we next investigated PICH localization in cells that had been simultaneously depleted of both kinases. Appropriate immunofluorescence markers indicate that the overall kinase activities of Plk1 and Aurora B are not influenced by each other (Fig. 25a+b, in collaboration with Ulf R. Klein). In this assay γ-tubulin was used as readout for Plk1 kinase activity and phospho-S7-CENP-A (pCENP-A)

for Aurora B kinase activity. In cells simultaneously depleted of Plk1 and Aurora B, PICH was absent from the KT/centromere and did not localize to chromosome arms (Fig. 25c).

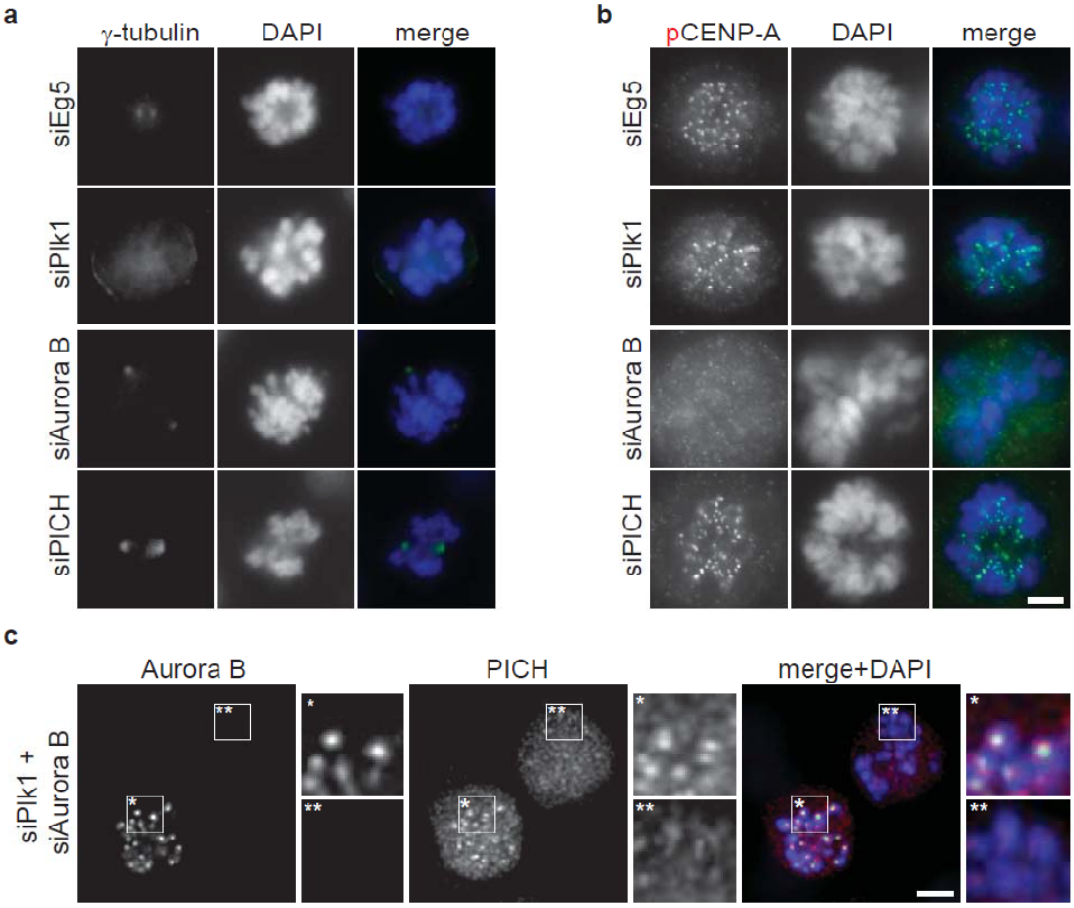



**Figure 25: Dependency of Aurora B and Plk1 kinase activities and their influence on PICH localization.**
**a** γ-tubulin staining (*green*) was assessed in HeLa cells depleted of Plk1 (or siEg5 as control), Aurora B or PICH. **b** Phospho-S7-CENP-A (pCENP-A) staining (*green*) was assessed in HeLa cells depleted of Plk1 (or siEg5 as control), Aurora B or PICH. DNA was stained with DAPI (*blue*). *Scale bar* in a and b, 5 µm **c** HeLa cells were simultaneously depleted of Aurora B and Plk1 by specific siRNA treatment and stained for the indicated antibodies. *Boxes* indicate regions of blow-up images. * denotes non-depleted cell, ** denotes depleted cell. Of note, PICH cannot localize to the chromosome arms in cells depleted of Aurora B. DNA was stained with DAPI (*blue*). *Scale bar*, 5 µm

This result implies that either PICH has to locate to the KT/centromere before it can be recruited to chromosome arms, or alternatively, that Aurora B is required for PICH spreading to the arms.

To test the first possibility, we mislocalized PICH from the KT/centromere region by the depletion of Hec1/Nuf2, before HeLa cells were additionally treated with the Plk1 inhibitor TAL, which normally recruits PICH to chromosome arms. Under conditions of Hec1 and Nuf2 knockdown and simultaneous treatment with TAL, PICH is recruited to chromosome

arms, arguing against the idea of KT/centromere recruitment of PICH as a prerequisite for chromosome arm spreading upon Plk1 inhibition (Fig. 26).

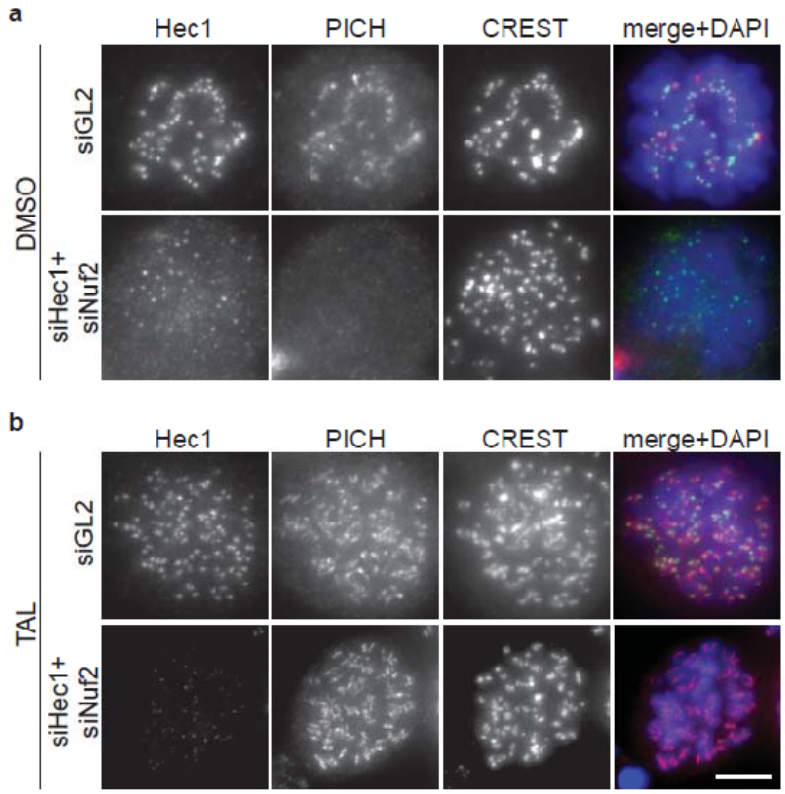



**Figure 26: PICH localization to chromosome arms.** HeLa cells depleted of Hec1 and Nuf2 were cycled form a thymidine block into mitosis in the presence of **a** DMSO (control) or **b** the Plk1 inhibitor TAL. Immunofluorescence was performed with specific antibodies directed against Hec1 (*green*) and PICH (*red*). CREST antibody staining (not shown in merge+DAPI) indicates KT localization of Hec1 and PICH. PICH localizes to chromosome arms upon TAL treatment irrespective of the presence of Hec1/Nuf2. DNA was stained with DAPI (*blue*). *Scale bar*, 5µm

In order to address the question if Aurora B kinase activity is required for PICH spreading to chromosome arms, we combined chemical inhibition of Plk1 and Aurora B. Aurora B kinase activity was inhibited by ZM447439 after the inhibition of Plk1 by TAL (see time course Fig. 27a). In contrast to single Plk1 inhibition, cells in which Plk1 and Aurora B were inhibited concurrently showed PICH localization to the KT/centromere but not to chromosome arms (Fig. 27b). It should be noted that PICH is restored to the KT/centromere after double inhibition of both kinases but is completely mislocalized after double depletion of Plk1 and Aurora B by siRNA (Fig. 25c). Furthermore, Plk1 localization to chromosome arms correlates with PICH localization, confirming the idea that PICH plays a major role in recruiting Plk1 to chromosome arms (Fig. 27b, Kurasawa and Yu-Lee, 2010; Santamaria et al., 2007).

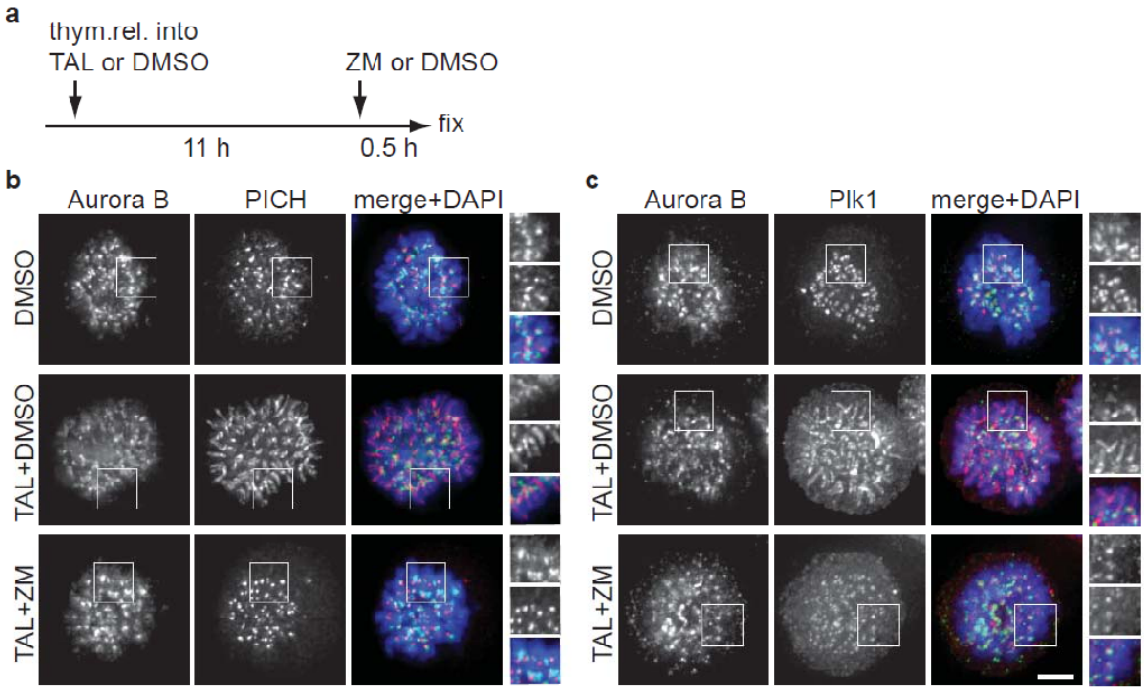



**Figure 27: Aurora B kinase activity is required for PICH localization to chromosome arms. a** Time course used in b and c. **b + c** HeLa cells were cycled from a thymidine block into mitosis in the presence of TAL and ZM447439 (or DMSO as control) and subjected to immunofluorescence microscopy using Aurora B (*green*) and PICH (*red*) specific antibodies. DNA was stained with DAPI (*blue*). *Boxes* indicate blown-up images. *Scale bar*, 5 µm

Collectively, these data show that these two kinases play antagonistic roles with regard to the arm localization of PICH. Aurora B kinase activity is required for PICH recruitment to chromosome arms, whereas Plk1 kinase activity is needed for the removal of PICH from the arms. Furthermore and most interestingly, chromosome arm localization of PICH seems to be differently regulated compared to the KT/centromere recruitment of PICH. The fact that PICH localization was still detectable at the KT/centromere after simultaneous treatment of TAL and ZM447439 supports the observation that PICH recruitment to the KT/centromere depends on the presence of Aurora B, yet is independent of Aurora B kinase activity.

### 1.9. PICH is phosphorylated by Aurora B *in vivo* and *in vitro*

As Aurora B kinase inhibition showed a drastic influence on PICH localization to chromosome arms we next asked if PICH, in addition to being a Plk1 substrate, can also be phosphorylated by Aurora B. HeLa cells were arrested in mitosis by nocodazole treatment and compared to cells treated with DMSO for control, TAL to inhibit Plk1 and/or ZM447439 for Aurora B kinase inhibition (see time course in Fig. 28a) and analyzed by Western blotting. Independent of the duration of the treatment and in agreement with previous reports

(Baumann et al., 2007; Santamaria et al., 2007), inhibition of Plk1 by TAL resulted in a significant increase in the electrophoretic mobility of PICH (Fig. 28b, *lane 3* and *4*) compared to DMSO treatment (Fig. 28b, *lane 1*). Inhibition of Aurora B kinase induced an electrophoretic mobility shift of PICH that was not as striking as for Plk1 inhibition, yet highly reproducible (Fig. 28b, *lane 5* and *6*). Simultaneous inhibition of both kinases showed complete dephosphoylation of PICH comparable to the treatment with calf intestine phosphatase (CIP, Fig. 28b, *lane2, 7, 8, 9* and *10*), which was added as further control. An antibody directed against phospho-serine 10 of histone H3 (pS10H3) was used to detect successful Aurora B kinase inhibition.

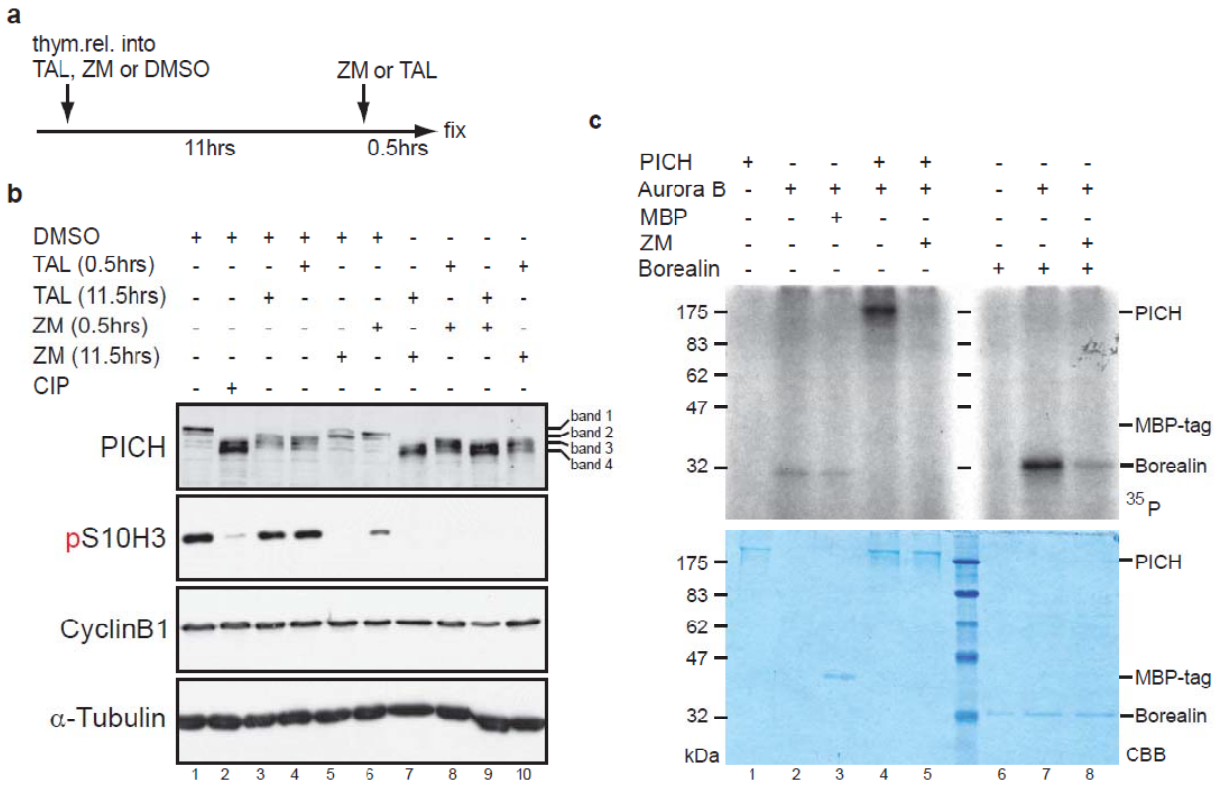



**Figure 28: Aurora B phosphorylates PICH *in vivo* and *in vitro*. a** Schematic time course used in b and c. **b** Cells were harvested by mitotic shake-off and cell lysates were analyzed by Western blotting. Both, TAL and ZM447439 addition influenced the phosphorylation status of PICH. *Band 1* displays highly phosphorylated PICH, *band 2* and *band 3* an intermediate phosphorylated form of PICH and *band 4* runs at the height of unphosphorylated PICH. Phospho-S10-histone H3 (pS10H3) is used as read-out for Aurora B kinase activity. Cyclin B1 level indicates the mitotic status and α-tubulin level serves as loading control. **c** The kinase assay shows that the recombinantly purified Aurora B/INCENP complex phosphorylates PICH *in vitro*. Proteins were analyzed by autoradiography ($^{35}$P) and Coomassie Brilliant Blue staining (CBB). MBP-tagged PICH was readily phosphorylated by Aurora B/INCENP (*lane 4*). His-Borealin serves as a positive control (*lane 7*). Addition of ZM447439 outlines the specificity of the phosphorylations (*lane 5* and *8*).

This suggests that PICH is not only phosphorylated by Plk1 *in vivo* but also by Aurora B. The fact that inhibition of both Plk1 and Aurora B showed the same shift as CIP treatment indicates that the major phosphorylations on PICH are mediated by these two kinases, in addition to the previously described requirement for Cdk1 phosphorylation for Plk1 binding.

To analyze PICH phosphorylation *in vitro*, recombinantly purified GST-INCENP/His-Aurora B complex (kindly provided by Ulf R. Klein) was incubated with bacterially expressed and purified MBP-PICH or His-Borealin as positive control. Both PICH and Borealin were readily phosphorylated by Aurora B in this assay. Addition of the Aurora B kinase inhibitor prevented the phosphorylation, attesting to the specificity of this approach (Fig. 28c). Together, these data show PICH to be an Aurora B kinase phosphorylation target. The apparent contradiction of PICH being a substrate of Aurora B but not requiring its kinase activity for KT/centromere localization can be explained by the existence of two different mechanisms, one recruiting PICH to the KT/centromere region (kinase independent) and another recruiting PICH to chromosome arms (kinase dependent). For details see section 1 and 2 in the Discussion.

### 1.10. PICH localization seems not to be required for Mad2 recruitment to KTs

The SAC has been speculated to sense two ultimately intertwined events, namely MT-KT attachment and/or the generation of tension between sister KTs when bi-orientation of chromosomes has been achieved. The CPC seems to constitute the tension sensing part of the SAC and the above data show that the localization of the proposed spindle checkpoint protein PICH depends on the CPC. Accordingly, cells depleted of the CPC show a SAC-dependent mitotic arrest in response to nocodazole (no MT-KT attachment) but override a taxol induced mitotic arrest (MT attachment but no generation of tension between sister KTs). The underlying mechanism has not been elucidated so far. Strikingly, PICH as well as the checkpoint protein Mad2 are lost from the KT/centromere in CPC-depleted cells (Vigneron et al., 2004). In addition, PICH knockdown also displaces Mad2 from the KT (Baumann et al., 2007). Because of this, we decided to analyze if the unusual behavior of mitotic arrest in CPC- depleted cells in the presence of nocodazole might be explained by the levels of PICH localized at the KT/centromere. Together with the localization of PICH we aimed to further elucidate how Mad2 is co-targeted to the KT.

To this end, HeLa cells were treated with control (GL2) or Aurora B siRNA before additional treatment with taxol or nocodazole, respectively. In control cells, PICH localization was as described above after treatments with either MT poison. Mad2 was detected at the KTs in

nocodazole but not in taxol treated cells, as expected (Fig. 29a). Simultaneous depletion of Aurora B and INCENP resulted in the mislocalization of PICH after taxol and nocodazole treatment. Mad2, previously described to be mislocalized by the depletion or inhibition of Aurora B (Kallio et al., 2002; Hauf et al., 2003; Ditchfield et al., 2005; Gadea and Ruderman, 2005) was found at the KTs in nocodazole treated cells but remained absent from KTs in this assay when taxol was added (Fig. 29b). This suggests that the proposed spindle checkpoint function of PICH is not linked to Mad2 recruitment to the KTs.

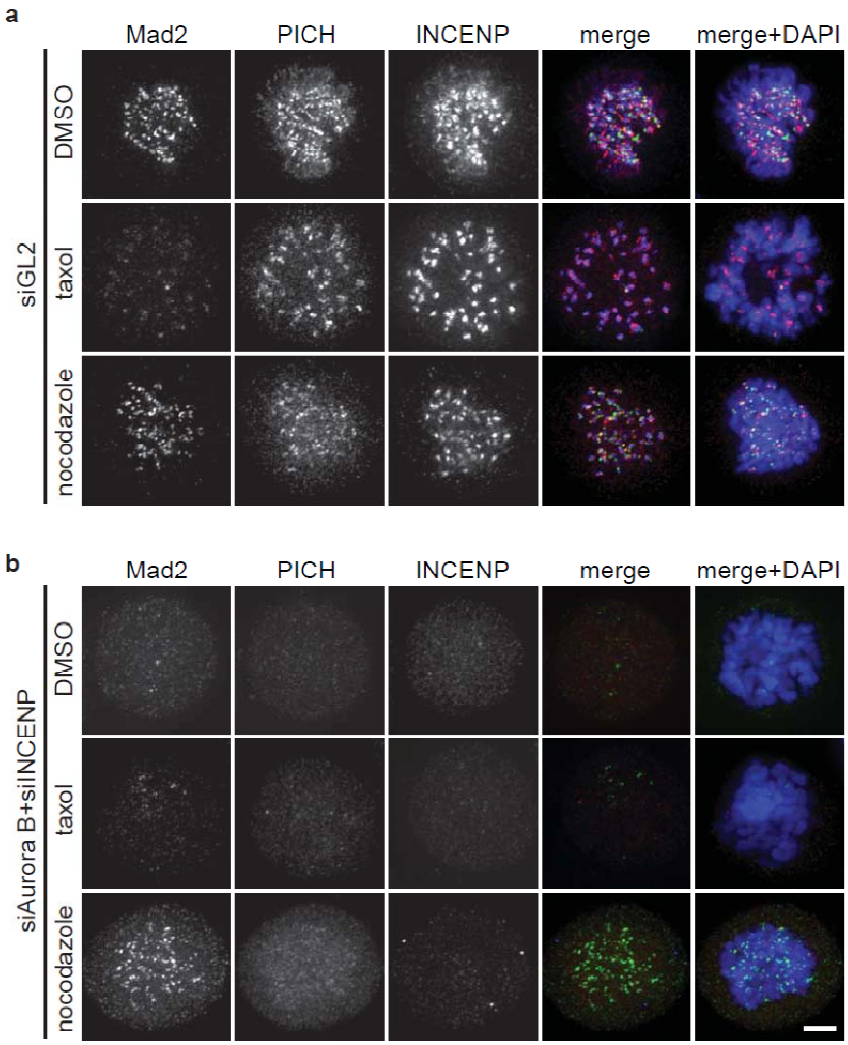



**Figure 29: Mad2 but not PICH is re-directed to KTs in Aurora B-depleted cells that have been treated with nocodazole. a** HeLa cells were treated with control siRNA oligonucleotides (siGL2) or **b** simultaneously depleted of Aurora B and INCENP by siRNA for 48 h and treated with DMSO (control), taxol or nocodazole, respectively, 2 h before fixation. Mad2 (*green*), PICH (*red*) and INCENP (not shown in merge+DAPI) localization was monitored by immunofluorescence staining. DNA was visualized by DAPI staining (*blue* in merge+DAPI). *Scale bar*, 5 µm

### 1.11. Conclusion Part 1

The DNA-dependent ATPase and proposed spindle checkpoint protein PICH was shown here to require the presence of the CPC and the Ndc80 complex for its KT/centromere localization. Both complexes have been shown to act independently in the recruitment of PICH and a direct interaction between these complexes and PICH was not detected. Most strikingly, PICH recruitment to the KT/centromere was dependent on the CPC but did not require Aurora B kinase activity, a behavior that has not been reported for any other KT/centromere associated protein so far. Moreover, the INCENP$^{F825A/F837A}$ mutant that cannot bind to Aurora B did not rescue PICH localization, indicating that the Aurora B protein is essential to target PICH to the KT/centromere. Interestingly, and in contrast to the Aurora B kinase activity-independent recruitment of PICH to the KT/centromere, the chromosome arm localization of PICH seems to depend on Aurora B kinase activity. Although a direct interaction between Aurora B and PICH has not been found, PICH represents a novel phosphorylation target of Aurora B *in vivo* and *in vitro*. The actual phosphorylation sites and their specific function remain elusive.

The PICH interaction partner Plk1, which was shown to regulate chromosome arm recruitment of PICH, was found to localize to KTs in an Aurora B kinase activity-dependent manner. We thus conclude that Plk1 acts downstream of Aurora B on PICH recruitment to the KT/centromere and that both kinases have an antagonistic effect on chromosome arm localization of PICH.

Remarkably, PICH was absent from the KT/centromere in Aurora B-depleted cells that were treated with nocodazole. In contrast, Mad2 was efficiently recruited to the KTs under these conditions. Thus, Mad2 KT localization is likely to explain the mitotic arrest seen under conditions of Aurora B depletion and addition of nocodazole. Furthermore, these results imply that the suggested function of PICH in the SAC cannot be based on the recruitment of Mad2 to the KT/centromere. This rather surprising result prompted us to re-investigate the role of PICH in the SAC in more detail, as described in the next section.

## 2. Re-examination of the proposed SAC function of PICH and Tao1

### 2.1. Different PICH specific siRNA oligonucleotides abrogate the SAC

To carefully re-explore the function of PICH in the SAC, siRNA-mediated knockdown experiments and detailed analysis by live-cell imaging were performed. PICH could effectively be depleted from HeLa cells using any of the three previously published siRNA oligonucleotides, here referred to as PICH-1, PICH-2 (Baumann et al., 2007) and PICH-CC (Leng et al., 2008). Live-cell imaging performed on HeLa cells stably expressing histone H2B-GFP showed that all three siRNA treatments also caused virtually identical mitotic phenotypes. Compared to control siRNA (siGL2) treated cells, PICH-depleted cells failed to form proper metaphase plates and displayed premature onset of anaphase (Fig. 30a). Moreover, cells depleted of PICH failed to undergo cell cycle arrest in response to nocodazole (Fig. 30b), confirming previous results (Baumann et al., 2007). Taken at face value, the results observed with these three independent siRNA oligonucleotides suggested a role for PICH in SAC function.

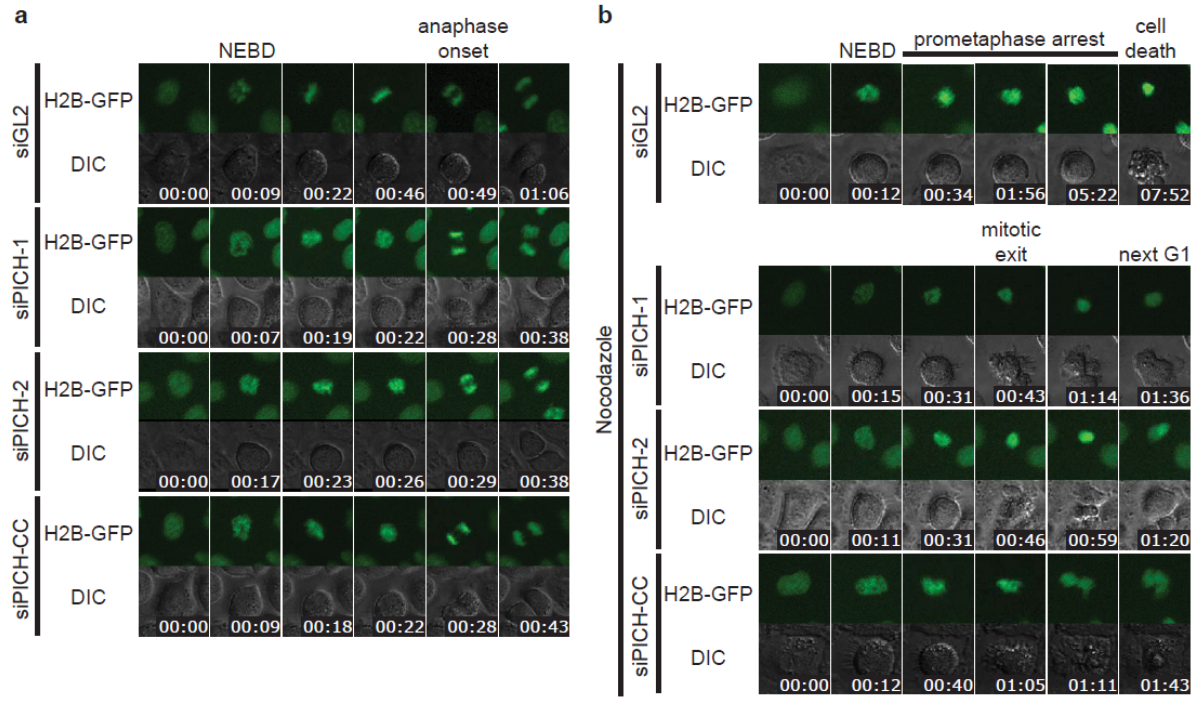



**Figure 30: Cell treated with PICH siRNA oligonucleotides display a defective SAC.** HeLa cells stably expressing histone H2B-GFP were treated with three different siRNA duplexes targeting PICH (siPICH-1, siPICH-2, and siPICH-CC), synchronized by a single thymidine block and analyzed by time-lapse microscopy 48 h after transfection. **a** Selected frames from representative live-cell movies show cells that traverse mitosis after treatment with control (GL2) or PICH-directed siRNA. *Upper panels* show GFP fluorescence; *lower panels* show differential interference contrast (DIC). **b** Cells were treated with nocodazole and filmed as described. Note that PICH-depleted cells fail to sustain the nocodazole-induced checkpoint arrest that is seen in control siRNA (siGL2) treated cells. All time stamps represent hours:minutes.

To strengthen the conclusion that PICH is the target of the siRNA duplexes used, rescue experiments were performed to examine whether normal mitotic timing could be restored (in collaboration with Lily H.-C. Wang). PICH-1 siRNA treated cells were simultaneously transfected with siRNA resistant PICH constructs tagged with mCherry. The average time from nuclear envelope breakdown (NEBD) to anaphase onset was 43.8 min in control cells (siGL2+mCherry). PICH-depleted cells expressing mCherry vector alone expeditiously initiated anaphase within an average time of 26.8 min (Fig. 31). In contrast, the overexpression of mCherry-tagged PICH wild type (WT) successfully restored the mitotic timing to 42.6 min, although some cells died, as previously described (Baumann et al., 2007). Hence, the concordant phenotype produced by three independent siRNA oligonucleotides and a successful rescue initially supported our hypothesis that PICH plays an essential role in SAC signaling.

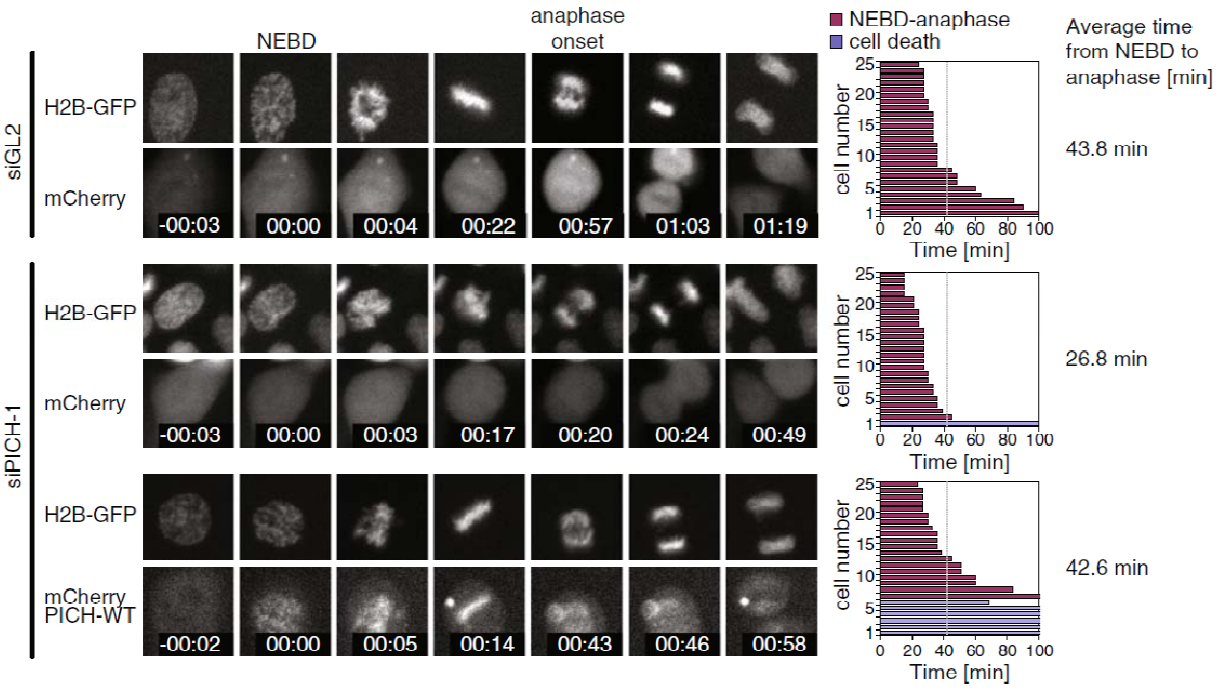



**Figure 31: Transient overexpression of PICH in PICH-depleted cells restores mitotic timing.** HeLa cells stably expressing histone H2B-GFP were transfected simultaneously with the indicated siRNA duplexes and plasmids and analyzed by time-lapse microscopy. The average time from NEBD to anaphase onset was analyzed in mCherry-positive cells. *Left panels* show selected single-plane images from movies, and time stamps indicate hours:minutes. *Right panels* show representative fate maps of 25 individual cells (the whole experiment being repeated independently three times). *Red columns* indicating the time span from NEBD to anaphase onset and *blue columns* indicating cells that died in interphase during imaging. Average times from NEBD to anaphase onset are indicated to the *right*.

## 2.2. Unexpected results question the fundamental role of PICH in SAC signaling

While investigating the function of PICH in more detail, several observations challenged the proposed conclusion that PICH plays a key role in checkpoint signaling. A first unexpected result emerged when a 293T cell line (293T TREX-shPICH) was generated, which allowed the tetracycline-inducible expression of a short hairpin RNA (shRNA), encoding the PICH-1 siRNA sequence. Three stable clones were obtained, all of which showed efficient PICH depletion after 72 h of shRNA expression. This depletion was reversible, as PICH protein was partially restored upon removal of tetracycline and incubation of cells in tetracycline-free medium for 72 h (Fig. 32a). To our surprise, none of the three 293T TREX-shPICH cell lines showed any sign of SAC failure, micronucleation (data not shown) or mislocalization of the checkpoint protein Mad2 (Fig. 32c), although PICH was efficiently depleted after tetracycline treatment (Fig. 32b).

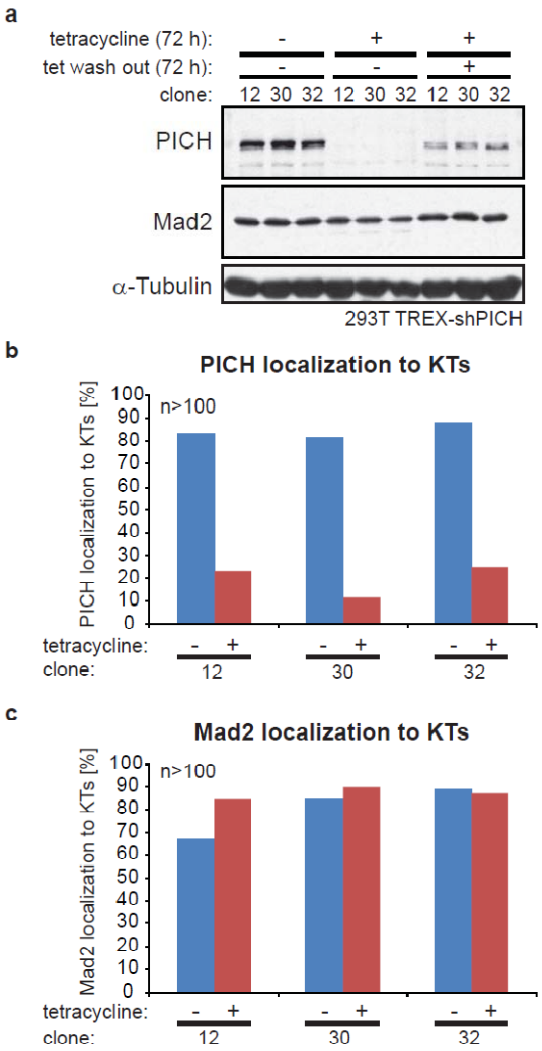



**Figure 32: PICH is not essential for SAC activity in 293T TREX cells.** **a** After selection of three different clones (nos. 12, 30, 32) of 293T TREX cells allowing the inducible expression of a PICH-directed shRNA (modeled after PICH-1 siRNA), PICH and Mad2 protein levels were monitored by Western blotting. α-Tubulin serves as a loading control. In parallel, KT localization of PICH and Mad2 was examined by immuno-fluorescence microscopy. **b** *Bar graph* shows quantitative analysis of PICH localization to KTs before and after 72 h tetracycline induction (n > 100). **c** *Bar graph* shows quantitative analysis of Mad2 localization to KTs before and after 72 h tetracycline induction (n > 100).

These results indicate the existence of cell-type specific differences in HeLa and 293T cells to PICH depletion, questioning the idea of PICH being an essential component of the SAC in all cell types.

In an independent set of experiments, we tried to restore SAC activity in PICH-depleted HeLa cells by re-expressing PICH protein at physiological levels. Specifically, we examined the consequences of depletion of PICH from a HeLa cell line stably expressing the LAP-tagged mouse homolog of PICH (Hutchins et al., 2010; Poser et al., 2008, in collaboration with Lily H.-C. Wang). Mouse PICH (Ercc6L) shares extensive sequence similarity with its human ortholog (72% amino acid identity; 81% amino acid similarity). However, expression of LAP-mouse PICH failed to restore SAC functionality in PICH-1 siRNA treated HeLa cells, as indicated by extensive micronucleation (Fig. 33a) and accelerated mitosis (Fig. 33b). Western blot analysis revealed that LAP-mouse-PICH was clearly resistant to PICH-1 siRNA treatment. In fact, LAP-mouse PICH showed enhanced expression upon depletion of endogenous human PICH, suggesting the existence of a feedback mechanism controlling total PICH protein levels (Fig. 33c, in collaboration with Lily H.-C. Wang).

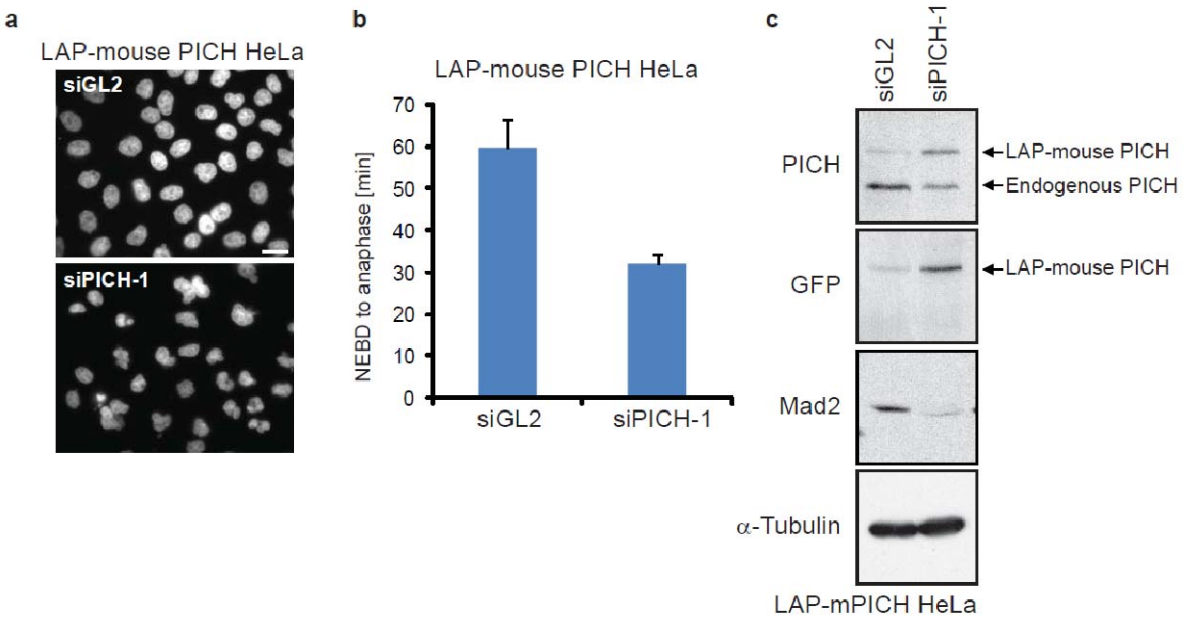



**Figure 33: LAP-mouse PICH fails to rescue PICH-1 siRNA phenotype. a** Morphological examination of the nuclei of HeLa cells expressing LAP-mouse PICH after 48 h of siRNA treatment with GL2 (control) or PICH-1 siRNA, respectively. DNA was visualized by DAPI staining. *Scale bar*, 10 µm. **b** Mitotic timing in siRNA-treated HeLa cells expressing LAP-mouse PICH. Cells were transfected with the indicated siRNA duplexes and analyzed by live-cell imaging. The *bar graph* indicates the average time from NEBD to anaphase onset. Data were collected from three experiments (20 cells each). *Error bars* indicate standard errors. **c** Cells were transfected with the indicated siRNA duplexes for 48 h and cell lysates probed by Western blotting with antibodies against PICH, GFP (to selectively visualize LAP-mouse PICH), Mad2, and α-Tubulin.

These rather unexpected results suggest that either mouse PICH is not able to provide SAC functionality in human cells, that LAP tagging interferes with protein functionality, or that PICH is not the only target of PICH-1 siRNA.

### 2.3. Mad2 protein and mRNA levels are significantly reduced upon depletion of PICH

Earlier studies suggested that depletion of PICH by siRNA affects Mad2 localization to KTs rather than Mad2 protein levels (Baumann et al., 2007). In the course of the above studies, we noticed that treatment of HeLa cells with the siPICH-1 oligonucleotide caused a reduction of Mad2 protein (Fig. 33c). This prompted us to re-examine the expression of Mad2 protein in response to various PICH siRNA oligonucleotides. To our surprise, depletion of PICH by all three previously published PICH-directed siRNAs showed a significant and highly reproducible, albeit partial reduction of Mad2 protein levels (Fig. 34a). The reduction of Mad2 protein was independent of the cell cycle stage, since identical results were obtained regardless of whether cells were arrested by thymidine or collected by mitotic shake off from a MG132 arrest (data for siPICH-1 shown in Fig. 40). To rule out general effects related to siRNA transfection, cells were treated with siRNA oligonucleotides targeting several other genuine SAC components. Depletion of these proteins did not cause a reduction of Mad2 protein levels (Fig. 34b).

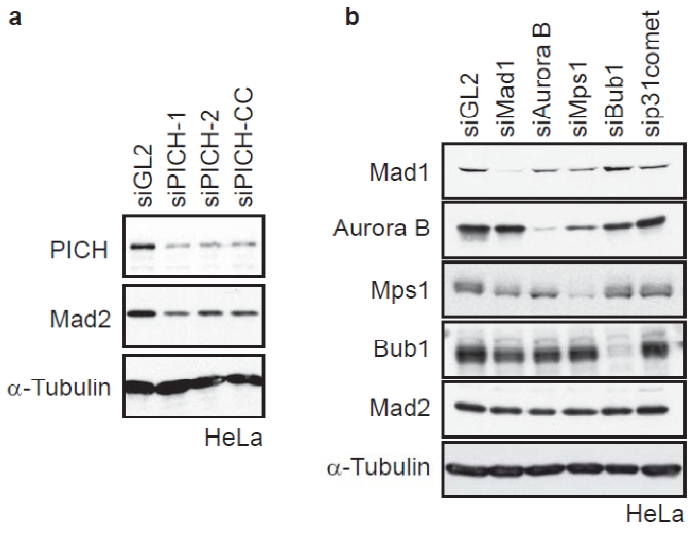



**Figure 34: Effect of PICH siRNA treatment on Mad2 expression. a** HeLa cells were transfected with the indicated siRNA duplexes and levels of PICH, Mad2, and α-Tubulin (loading control) were monitored by Western blotting. **b** For control, various checkpoint proteins were depleted by siRNA, as indicated, and protein levels determined by Western blotting.

This demonstrates that the observed reduction of Mad2 levels in PICH-treated cells is not simply a consequence of SAC abrogation or a general effect related to siRNA transfection. If the previously observed loss of Mad2 protein from KTs in PICH-depleted cells reflected a depletion of Mad2 protein rather than a selective disruption of the Mad1-Mad2 interaction at KTs, we reasoned that it might be possible to restore Mad2 localization to KTs by overexpression of exogenous Mad2 protein in PICH siRNA treated cells. This was indeed the case, as the Mad2-GFP fusion protein localized to KTs in a PICH-1 siRNA background (Fig. 35a). As control, overexpressed Mad2-GFP did not localize to KTs in cells depleted of Aurora B (Fig. 35b), consistent with previous reports (Ditchfield et al., 2003; Vigneron et al., 2004). Through this line of evidence, we support the conclusion that the PICH siRNA phenotype reported previously might reflect a change in Mad2 abundance rather than localization.

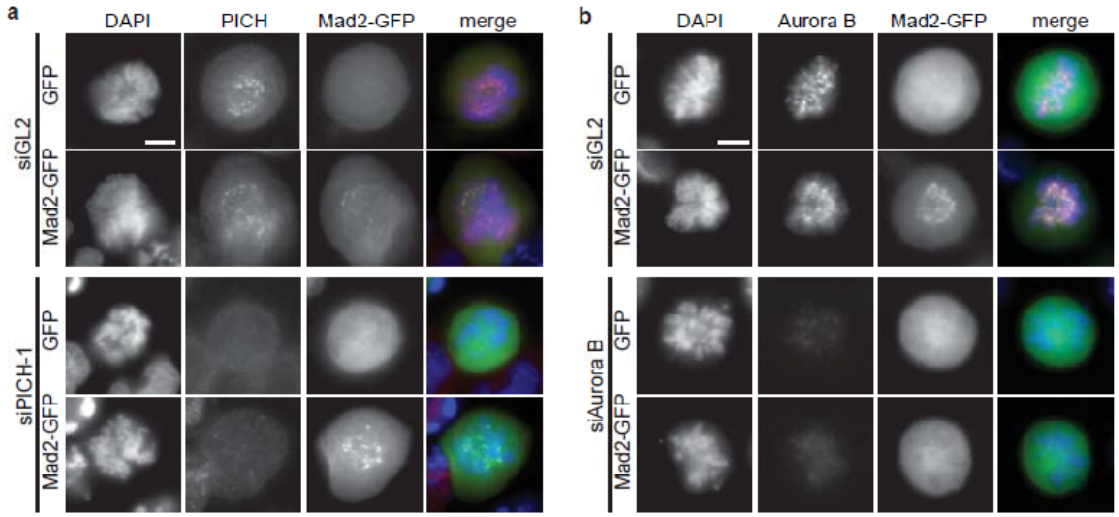



**Figure 35: Overexpressed Mad2-GFP localizes to KTs in cells treated with PICH-1 siRNA.** Immuno-fluorescence microscopy images of prometaphase cells after treatment with the indicated siRNAs for 48 h and transfection (during the last 24 h) with either empty-GFP vector or GFP-tagged Mad2. Cells were fixed and stained with **a** PICH or **b** Aurora B antibody (*red*), respectively. DNA was stained with DAPI (*blue)*. *Scale bars*, 5 μm.

To investigate the mechanism underlying the reduction in Mad2 protein levels upon PICH depletion, we next examined whether this reduction was also reflected at the mRNA level. To this end, PICH, Mad1, and Mad2 mRNAs were measured by quantitative real-time polymerase chain reaction (qRT-PCR). As control, HeLa cells were either depleted of GL2 or Mad1. As expected, all three PICH siRNAs caused almost identical knockdown efficiencies with regard to PICH mRNA. Interestingly, all three PICH siRNAs display a significant

decrease of Mad2 mRNA when compared to control siRNAs, whereas Mad1 mRNA was not detectably affected (Fig. 36).

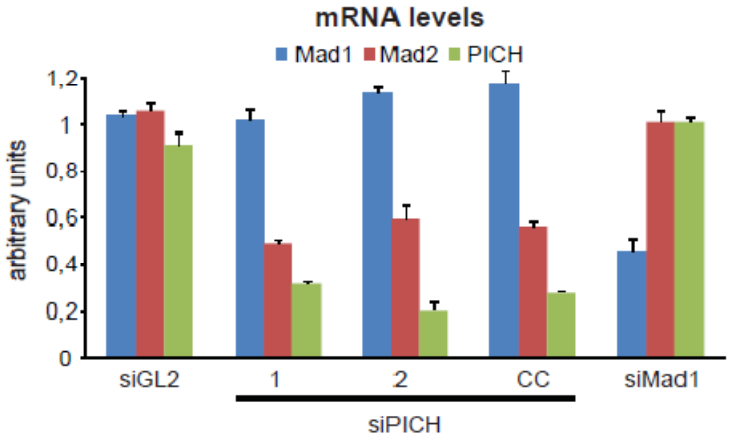



**Figure 36: Mad2 mRNA levels are significantly decreased in PICH-depleted cells.** HeLa cells were transfected with the indicated siRNAs and levels of Mad1 (*blue*), Mad2 (*red*), and PICH (*green*) mRNA were measured by qRT-PCR. *Bar graphs* show mRNA levels averaged from three independent experiments (arbitrary units, AU), and *error bars* represent standard errors.

These results underline the suggestion that treatment of HeLa cells with any of the three previously published PICH siRNA oligonucleotides reduces not only PICH but also Mad2 mRNA levels. Thus, the formerly observed loss of checkpoint functionality might actually reflect a partial but significant reduction of cellular Mad2 protein. This further implies that either PICH protein somehow regulates Mad2 expression or, alternatively, that the three PICH-directed siRNA oligonucleotides all display off-target effects that cause a lowering of Mad2 expression.

### 2.4. PICH remains cytoplasmic upon leptomycin B treatment

The idea of PICH being involved in the regulation of Mad2 expression (e.g. at the level of transcription) could possibly imply that PICH protein locates to the nucleus during interphase. Immunofluorescence studies however, showed PICH staining only in the cytoplasm (within limits of detection). To examine whether PICH might shuttle between the cytoplasm and the nucleus, the following experiment was performed. HeLa cells were treated with leptomycin B to inhibit nuclear export and analyzed by immunofluorescence for PICH and cyclin B (control) localization. Cyclin B was found to accumulate in the nucleus, whereas PICH antibody staining revealed that PICH still localizes to the cytoplasm after a 2 h treatment with leptomycin B (Fig. 37).

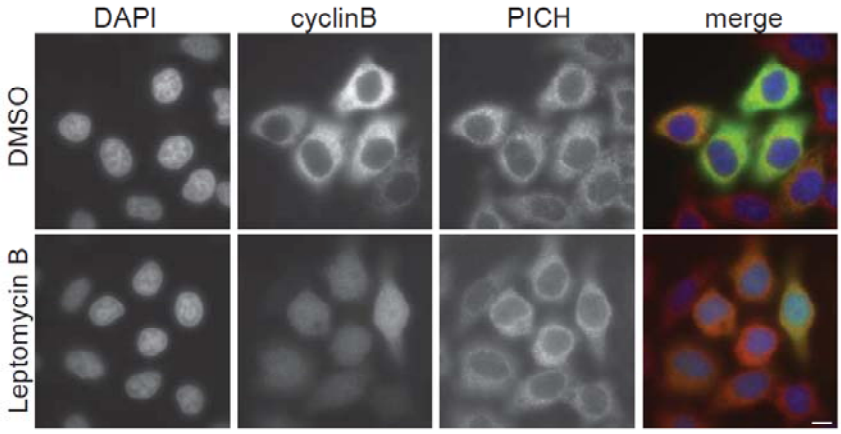



**Figure 37: Inhibition of nuclear export by leptomycin B treatment.** HeLa cells were treated with leptomycin B for 2 h and subjected to immunofluorescence microscopy. Cyclin B (control) is stained in *green*, PICH in *red* and DNA in *blue* (DAPI). *Scale bar*, 10 µm.

This result argues that PICH is not a shuttling protein and only found in the cytoplasm during interphase. Although this experiment does not completely exclude PICH from being located within the nucleus during interphase, it supports the idea that PICH is not important for the transcriptional control of the checkpoint protein Mad2.

### 2.5. Newly designed PICH siRNA oligonucleotides target PICH but not Mad2

In the next set of experiments, novel siRNA oligonucleotides that would effectively deplete PICH without lowering Mad2 mRNA and protein levels were designed. Indeed, four new siRNAs, referred to as PICH-3, PICH-4, PICH-5, and PICH-6, as well as a corresponding pool comprising all four duplexes (PICH-SP), were found to successfully deplete PICH. As shown in Fig. 38, PICH-3, PICH-4, and PICH-5 showed comparable efficiency to PICH-1, PICH-2, and PICH-CC siRNA in regard to PICH depletion, whereas PICH-6 and the pooled PICH-SP duplexes were slightly less efficient. To rule out a cell type specific effect, we also confirmed these results in U2OS and RPE-1 cells (data not shown). The kinetics of depletion was comparable between these oligonucleotides and similar results were obtained regardless of the duration of the siRNA treatment (data not shown, Hubner et al., 2010). Most importantly, and in striking contrast to PICH-1, PICH-2, and PICH-CC, none of the new PICH-directed siRNA oligonucleotides detectably affected Mad2 expression, regardless of whether Mad2 protein (Fig. 38a) or mRNA (Fig. 38b) was monitored. In comparison to the previously published PICH-1, PICH-2, and PICH-CC siRNA duplexes, none of the new oligonucleotides detectably interfered with KT localization of Mad2 (Fig. 38c+d).

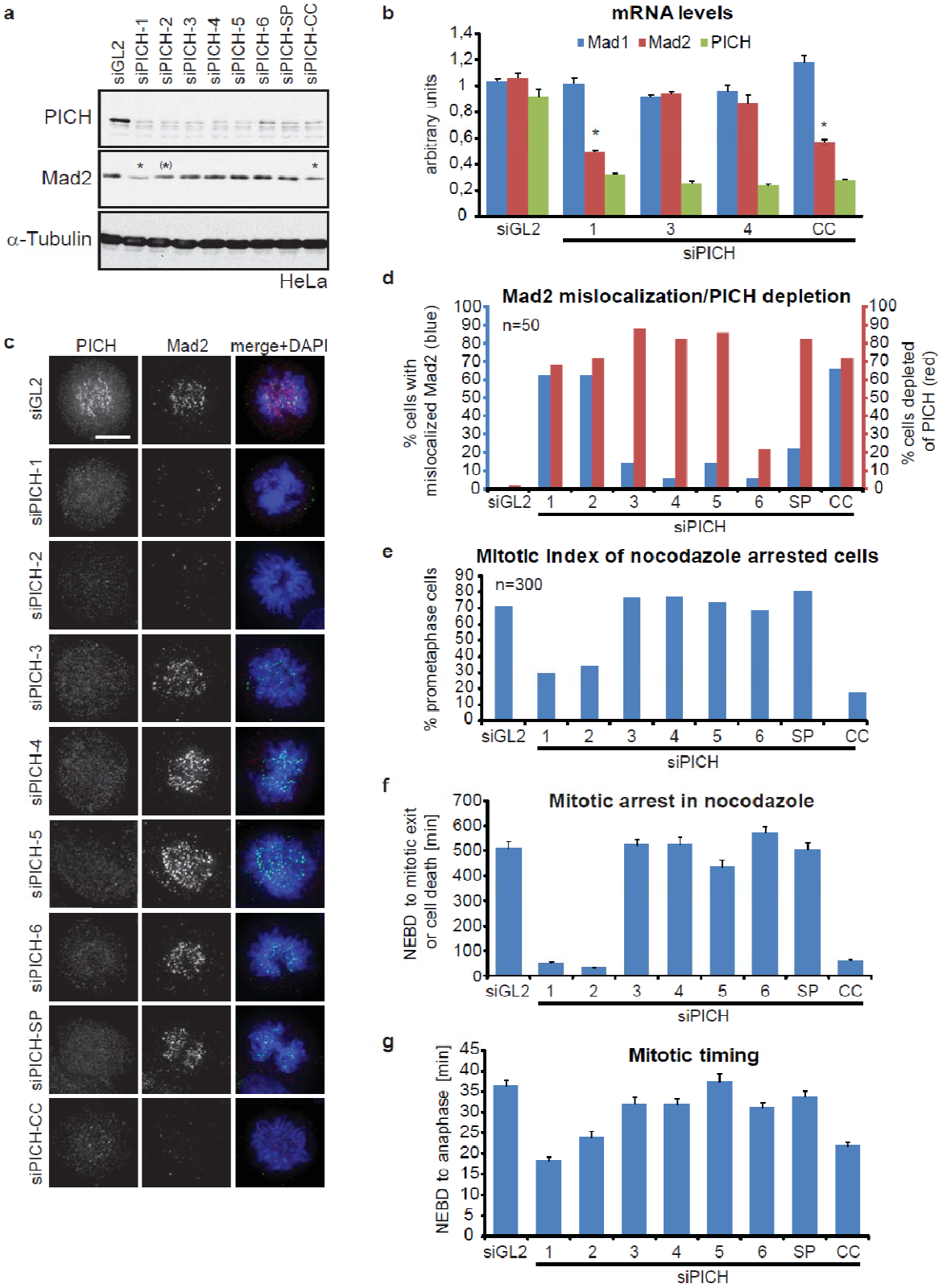



**Figure 38: Analysis of depletion efficiencies and phenotypes produced by different PICH-directed siRNA duplexes. a** HeLa cells were treated with indicated siRNAs before lysates were analyzed by Western blotting with the indicated antibodies. Note that Mad2 levels are reduced in response to siPICH-1, siPICH-2, and siPICH-CC (marked by *asterisks*). **b** HeLa cells were transfected with the indicated PICH siRNA oligonucleotides and arrested in thymidine prior to RNA purification and measurements of mRNA levels by qRT-PCR. *Histogram* illustrates relative mRNA levels of Mad1 (*blue*), Mad2 (*red*), and PICH (*green*; in AU). Note that Mad2 mRNA levels are significantly reduced in siPICH-1 and siPICH-CC (marked by *asterisk*). *Error bars* indicate standard

errors of three independent experiments. **c** Cells were treated with different PICH siRNAs and stained with antibodies directed against PICH (*red*) and Mad2 (*green*). DNA was stained with DAPI (*blue*). Note that Mad2 is displaced from KTs solely in response to siPICH-1, siPICH-2, or siPICH-CC oligonucleotides. **d** HeLa cells were treated as in **c**, and Mad2 mislocalization (displacement from KTs) was counted. The *graph* shows the percentage of mitotic cells that were depleted of PICH (*red bars*) as well as the percentage of cells showing mislocalization of Mad2 (*blue bars*) (n=50). **e** Following a 24 h thymidine block, HeLa cells were released for 16 h into nocodazole, and the percentage of mitotic cells was determined by visual inspection after DAPI staining. *Graph* shows the average from two independent experiments (n=300). **f** *Histogram* summarizes the results of live-cell time-lapse microscopy performed on cells that had been depleted of PICH by 48 h treatments with different PICH-directed siRNA duplexes and then treated with nocodazole during the last 16 h. *Error bars* indicate standard errors (n=40). **g** HeLa cells stably expressing H2B-GFP were transfected with the indicated siRNAs and analyzed by live-cell imaging. The *histogram* illustrates the elapsed average time from NEBD to anaphase onset (40 cells for each treatment), and *error bars* indicate standard errors.

The influence of the new PICH-directed oligonucleotides on the nocodazole induced SAC activation and the timing of mitotic progression were further examined. In addition to determine the mitotic index after nocodazole treatment in fixed cells (Fig. 38e), live-cell imaging was performed on HeLa cells stably expressing H2B-GFP in the presence (Fig. 38f) and absence of nocodazole (Fig. 38g). In striking contrast to cells treated with the previously described PICH siRNA duplexes (accelerated anaphase onset and abolished SAC activity), the mitotic timing (NEBD to anaphase onset) and the duration of the nocodazole induced SAC arrest in cells treated with the new PICH-directed siRNA oligonucleotides were similar compared to the GL2 siRNA-treated control cells. Thus, we consider that the originally described PICH siRNA duplexes interfere with SAC activity because they lower Mad2 transcript and protein levels through an off-target effect. Whether this off-target effect acts directly on the Mad2 transcript or through some unidentified factor remains unknown.

### 2.6. Re-evaluation of the role Tao1 kinase in the spindle checkpoint

In 2007, a genome-wide siRNA screen identified a requirement for the Tao1 kinase (also known as microtubule affinity-regulation kinase kinase (MARKK), Timm et al., 2003; Johne et al., 2008) in the spindle checkpoint (Draviam et al., 2007). Most interestingly, Tao1 depletion by siRNA was reported to cause a selective loss of Mad2 but not Mad1 from KTs (Draviam et al., 2007), very similar to the early data obtained after PICH siRNA (Baumann et al., 2007). Intrigued by this similarity, we originally suspected that PICH and Tao1 cooperate in a regulatory step to control Mad2 localization. However, in line with the obtained evidence that the apparent requirement for PICH in Mad2 localization is likely to reflect an off-target effect, we analyzed the Tao1 siRNA phenotype more carefully. To this end, different Tao1-directed siRNA oligonucleotides were compared for their efficiency to deplete Tao1, and their effects on Mad2 localization, protein and transcript levels, as well as their ability to abolish

SAC activity. This survey included the most effective previously published Tao1 siRNA oligonucleotide (Tao1-NCB3) as well four new oligonucleotides (Tao1-2 to Tao1-5) and a smart pool comprising Tao1-2 to Tao1-5 (Tao1-SP). Western blotting revealed that all Tao1-directed siRNAs exhibited a similar ability to deplete Tao1 from HeLa cells, and curiously, PICH-1 siRNA also lowered Tao1 levels (Fig. 39a). Surprisingly but most importantly, the already published siTao1-NCB3 oligonucleotide additionally caused a significant reduction of Mad2 protein, comparable to the reduction of Mad2 seen after PICH-1 or PICH-CC siRNA (Fig. 39a). In contrast, Mad2 levels remained unaffected upon treatment of cells with the other Tao1 siRNAs, except for Tao1-4, which produced a marginal effect. Similar results were obtained upon depletion of Tao1 from U2OS and RPE-1 cells (Fig. 39b). Quantitative Western blot analysis (three independent experiments; normalized to α-Tubulin) revealed that Tao1-NCB3 siRNA substantially lowered Mad2 protein levels. The other Tao1-directed siRNAs barely affected Mad2, although they depleted Tao1 with similar efficiency as Tao1-NCB3 (Fig. 39c). We observed that the KT localization of Mad2 was abolished only in cells treated with the Tao1-NCB3 siRNA but not in cells treated with any of the newly designed Tao1-directed siRNA oligonucleotides (Fig. 39d+e). Supporting our findings, a recently published study shows that Tao1 is unlikely to function as a spindle checkpoint component (Westhorpe et al., 2010). Currently we cannot explain the discrepancy between this observation and the previous report, indicating that Tao1 siRNA does not lower Mad2 levels (Draviam et al., 2007).

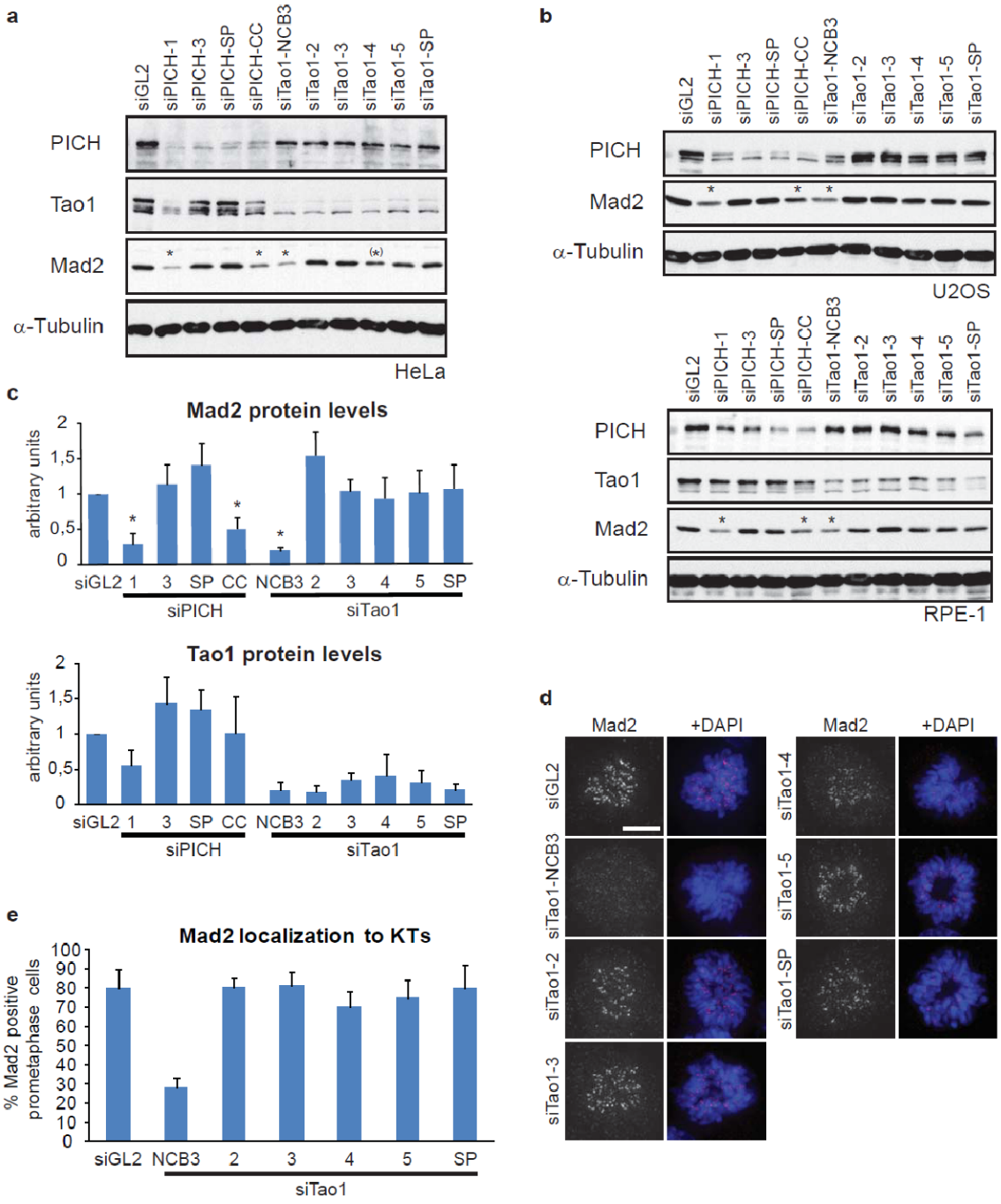



**Figure 39: Evaluation of protein knockdown efficiency, Mad2 localization, and SAC phenotype for different siRNA oligonucleotides targeting the Tao1 kinase. a** HeLa cells were transfected with the indicated siRNA duplexes, and lysates were probed by Western blotting with the indicated antibodies. *Asterisks* indicate the reduction of Mad2 levels by some but not all Tao1- and PICH-directed siRNAs. **b** Effects on Mad2 protein levels after depletion of PICH and Tao1 from U2OS and RPE-1 cells. U2OS (*upper panel*) and RPE-1 cells (*lower panel*) were treated with the indicated PICH- and Tao1-directed siRNA duplexes for 48 h, and the abundance of both the primary targets and Mad2 protein was analyzed by Western blotting. The *asterisks* indicate significant reduction of Mad2 protein levels. **c** Western blots from three individual experiments (in HeLa cells) were used for quantification of Mad2 and Tao1 protein levels. Band intensities were measured using the Aida Image Analyzer software. The *histograms* show the mean band intensities normalized against background and α-Tubulin, and *error bars* indicate standard errors. Note that Mad2 protein levels were significantly reduced in lysates from siPICH-1-, siPICH-CC-, and siTao1-NCB3-treated cells (marked by *asterisks*). **d** Cells were treated with different Tao1 siRNAs and stained with antibodies directed against Mad2 (*red*); DNA was stained with DAPI (*blue*). *Scale bar*, 5 µm. Note that Mad2 is displaced from KTs only in

response to Tao1-NCB3, but not other Tao1-directed duplexes. **e** Quantitative analysis of Mad2 localization in cells depleted of Tao1. HeLa cells were treated as described in d. The *graph* shows the percentage of prometaphase cells in which Mad2 localizes correctly to KTs; shown are the averages from three independent experiments with standard errors (n > 150).

Downregulation of Mad2 by PICH-1 and the Tao1-NCB3 siRNA oligonucleotides was independent of cell cycle stages as Mad2 protein was lost in thymidine-arrested interphase as well as MG132-arrested mitotic cell lysates (Fig. 40).

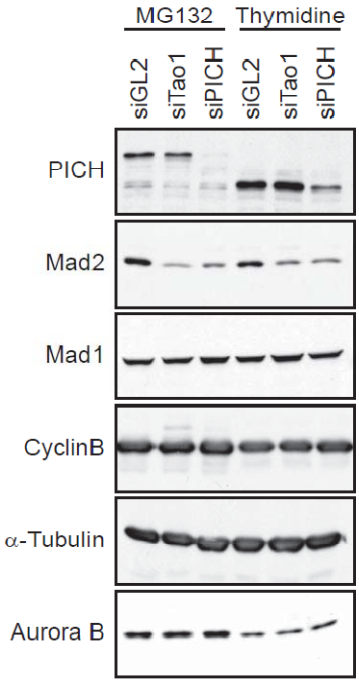



**Figure 40: PICH-1 and Tao1-NCB3 siRNA oligonucleotides reduce Mad2 protein levels independent of the cell cycle stage.** HeLa cells were transfected with PICH-1, Tao1-NCB3 or GL2 (control) siRNA for 48 h and arrested in interphase by a thymidine block for the last 24 h of siRNA treatment or released for 10 h from the thymidine block and treated with MG132 for the last 2 h of release before mitotic shake off. Cell lysates were subjected to Western blotting and analyzed with indicated antibodies.

Next, we used qRT-PCR to analyze the mRNA levels of Mad1 (control), Mad2, and Tao1 after treatment of HeLa cells with different siRNAs targeting Tao1 or PICH. Mad1 mRNA remained constant in all cases as expected. Tao1 mRNA levels were similarly reduced by all Tao1 siRNAs and, to a lesser extent, PICH-1 siRNA. Most importantly, Mad2 mRNA was significantly reduced upon treatment with siPICH-1 and siTao1-NCB3 siRNAs and, to a lesser extent, Tao1-4 siRNA. No changes in Mad2 mRNA levels were detectable in cells treated with siPICH-3, siTao1-2, siTao1-3, or control GL2 siRNA (Fig. 41).

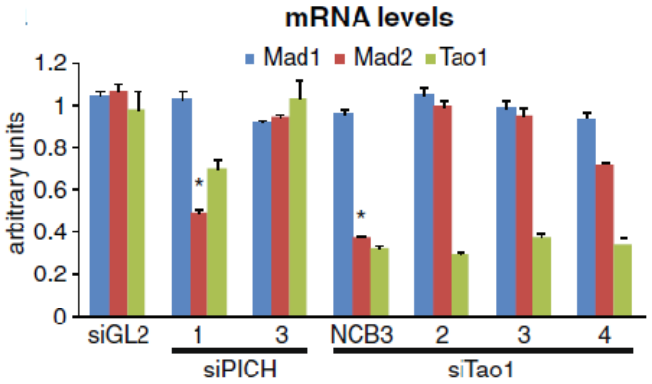



**Figure 41: *Histogram* illustrating mRNA levels after Tao1 siRNA treatment.** After treatment of HeLa cells with the indicated siRNAs, RNA was extracted for qRT-PCR measurements. *Bars* indicate relative mRNA expression levels of Mad1 (*blue*), Mad2 (*red*), and Tao1 (*green*; in AU); shown are averages from three independent experiments and standard errors. Significant reduction of Mad2 mRNA in PICH-1 and Tao1-NCB3 siRNA-treated cells is marked by *asterisks*.

In addition, the cellular phenotype of cells depleted of Tao1 by different oligonucleotides was analyzed. Cells that had been treated with the Tao1-NCB3 siRNA duplexes went through mitosis more rapidly (NEBD to anaphase; average 14 min) than siGL2-treated control cells (average 33 min). Compared to the other Tao1 siRNAs which did not show acceleration from NEBD to anaphase, the mitotic timing was slightly reduced in cells treated with the Tao1-4 siRNA (average 23 min; Fig. 42a). Parallel experiments showed an override of the nocodazole-induced SAC arrest in response to siTao1-NCB3 (average duration of arrest 25 min), consistent with the original report implicating Tao1 in SAC function (Draviam et al., 2007). A robust checkpoint arrest (often followed by cell death after 12-15 h) was seen upon depletion of Tao1 by siTao1-2 or siTao1-3. Cells treated with Tao1-4 siRNA showed a partial phenotype by usually exiting mitosis after about 4 h (Fig. 42b).

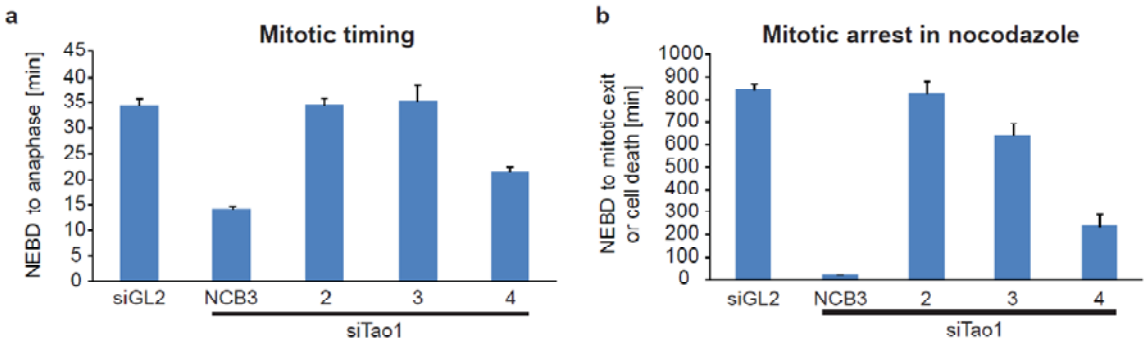



**Figure 42: *Histogram* illustrating the mitotic timing, and nocodazole response after Tao1-directed siRNA treatment.** HeLa cells stably expressing histone H2B-GFP were transfected with the indicated siRNAs and analyzed by live-cell imaging. **a** The *histogram* illustrates the elapsed average time from NEBD to anaphase onset (40 cells for each treatment), and *error bars* indicate standard errors. **b** *Histogram* summarizes the results of live-cell time-lapse microscopy performed on cells that had been depleted of Tao1 by 48 h treatments with different Tao1 siRNA duplexes and then treated with nocodazole during the last 16 h. *Error bars* indicate standard errors (n=40).

We noticed that the oligonucleotides giving the off-target effect were purchased from a different company than the newly designed siRNA duplexes. To rule out a company specific modification on the duplexes used, we compared identical oligonucleotide sequences purchased from the two companies (Qiagen and Dharmacon) by Western blotting (Fig. 43). The results confirmed that the off-target effect is sequence specific and can be observed in siRNA oligonucleotides purchased from both companies.

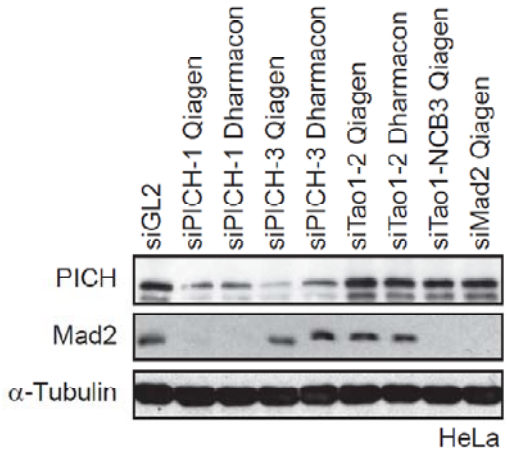



**Figure 43: Western Blot analysis of siRNA oligonucleotides from Qiagen and Dharmacon.** HeLa cells were treated with indicated siRNA oligonucleotides for 48 h and analyzed for PICH and Mad2 protein levels by Western blotting. α-Tubulin serves as loading control.

Collectively, we interpret the above data to suggest that the apparent role of Tao1 kinase in SAC signaling might in reality reflect an off-target effect. This effect is produced by particular Tao1-directed siRNA oligonucleotides either directly or indirectly on Mad2, similar to the situation described for certain PICH siRNA duplexes. Therefore, our data and the results published recently by Steven Taylor and coworkers (Westhorpe et al., 2010) do not support Tao1 being a component of the spindle checkpoint.

### 2.7. Rescue of PICH and Tao1 siRNA phenotypes by Mad2 expression from a bacterial artificial chromosome

The above data forced us to assume that the abrogation of the SAC in cells treated with particular PICH- and Tao1-directed siRNA oligonucleotides is caused by an off-target effect on Mad2. If this is the case, it should in principle be possible to rescue this mitotic phenotype by re-expressing Mad2. To test this prediction, we used a procedure for the introduction of genes from the mouse homologue tagged in bacterial artificial chromosomes (BACs) into human tissue culture cells. This allows the stable expression of genes under their own promoters at near physiological levels (Hutchins et al., 2010; Poser et al., 2008). The

expression of mouse Mad2 in the established cell line was characterized by immunofluorescence and it showed co-localization of human and mouse Mad2 (Fig. 44a) and correct localization to KTs (Fig. 44b). Depletion of PICH by the siPICH-1 oligonucleotide did not abolish mouse Mad2 recruitment to the KTs (Fig. 44c).

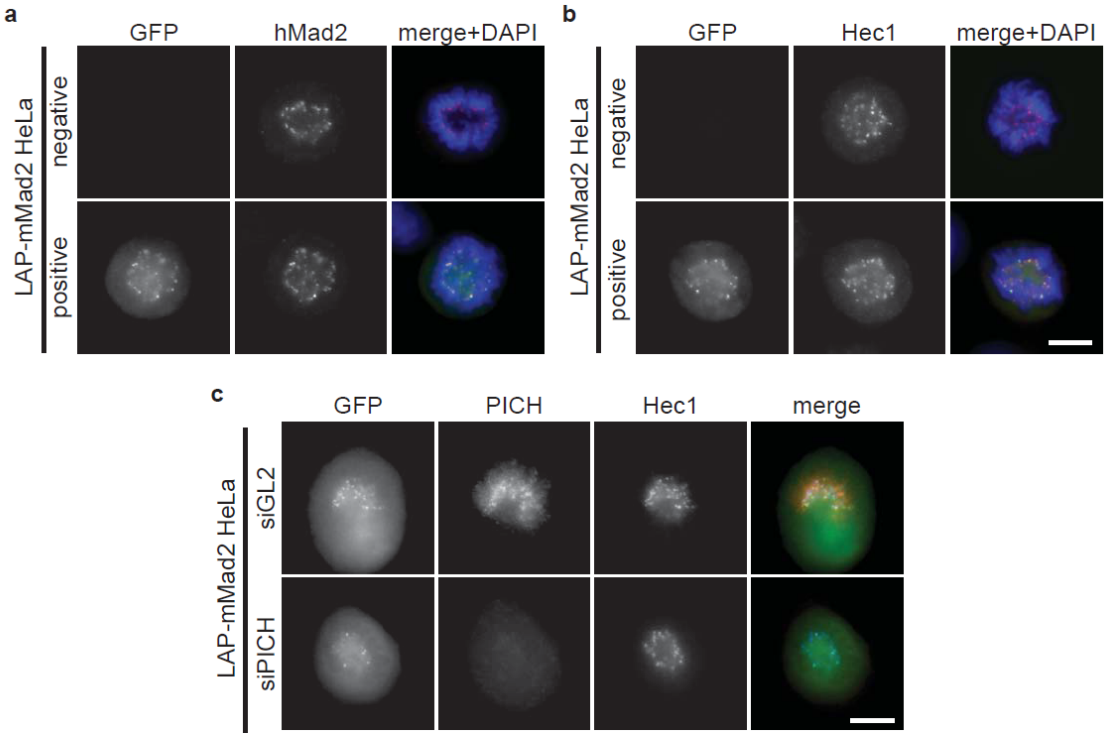



**Figure 44: Characterization of HeLa cells expressing LAP-tagged murine Mad2 by immunofluorescence.** **a** Cells were released from a single thymidine block for 10 h and fixed before staining with antibodies directed against human Mad2 (*red*). LAP-tagged murine Mad2 is shown in *green* (GFP) and DNA was stained with DAPI (*blue*). *Scale bar*, 10 µm. **b** LAP-mMad2 HeLa cells were treated with PICH-directed siRNA and subjected to immunofluorescence analysis with indicated antibodies. Note that after depletion of PICH, murine Mad2 properly localizes to KTs. *Scale bar*, 10 µm

In a next set of experiments a pool of HeLa cells expressing a LAP-tagged murine Mad2 was subjected to PICH, Tao1, or Mad2 siRNA, and SAC functionality was then examined by live-cell imaging (Fig. 45a). In this setup, GFP-positive cells (expressing murine Mad2) could directly be compared to GFP-negative cells (devoid of murine Mad2). In addition, we monitored the protein levels of PICH, Tao1, human Mad2, and murine Mad2 (detected by a GFP-specific antibody) by Western blotting (Fig. 45b).

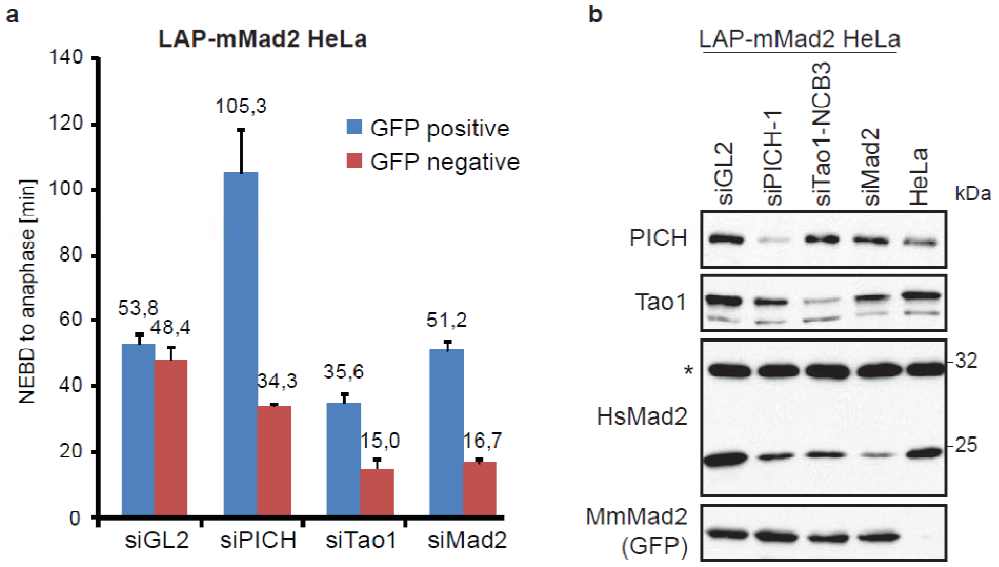



**Figure 45: Rescue of PICH-1 and Tao1-NCB3 siRNA phenotypes by LAP-mouse Mad2. a** Mitotic timing in siRNA-treated HeLa cells expressing LAP-mouse Mad2. Cells were transfected for 48 h with the indicated siRNA duplexes and analyzed by live-cell imaging. In these experiments, GFP-positive cells (expressing mouse Mad2) and GFP-negative cells (not expressing mouse Mad2) were monitored in parallel. The *bar graph* indicates the average time from NEBD to anaphase onset. Data were collected from four experiments (20–25 cells each), and *error bars* indicate standard errors. **b** Lysates were prepared from cells treated as in **a** and analyzed by Western blotting with the indicated antibodies. The *lane* to the *right* represents a lysate prepared from the parental HeLa (Kyoto) cell line that lacks the BAC expressing murine Mad2. *Asterisk* denotes a cross-reactive band that serves as loading control

As expected, the mitotic timing (NEBD to anaphase onset) was accelerated in GFP-negative cells subjected to PICH-1, Tao1-NCB3, or Mad2 siRNA, when compared to siGL2-treated control cells. The mitotic timing of GFP-positive cells was similar in cells treated with GL2 control or Mad2 siRNA, attesting the ability of murine Mad2 to compensate for the depletion of endogenous human Mad2. In the case of PICH-1 siRNA, GFP-positive cells showed a delay in the traverse of mitosis, suggesting that loss of PICH in cells harboring near physiological levels of Mad2 actually triggers a mitotic delay. Tao1-NCB3 siRNA-treated cells expressing murine Mad2 exited mitosis almost as fast as the GL2 control. Furthermore, we note that all GFP-positive cells (murine Mad2-expressing) showed a prolonged arrest in response to nocodazole, regardless of whether they were treated with PICH-1, Tao1-NCB3, or (human) Mad2 siRNA (data not shown). Collectively, these data make a strong case that the observed reduction of Mad2 levels is the main reason for the abrogation of SAC activity in the PICH- and Tao1-siRNA-treated cells.

### 2.8. Uncovering of a regulatory influence of Plk1 on Mad2 function

A critical role for PICH and Tao1 in the SAC had originally been supported not only by consistent siRNA phenotypes but also by apparently successful rescue experiments (this study Fig. 31, Draviam et al., 2007). SAC failure by Tao1-NCB3 siRNA could successfully be rescued by an active but not inactive kinase, strongly supporting a role for Tao1 in the SAC (Draviam et al., 2007). However, the published evidence suggests that this rescue involved overexpression of siRNA-resistant Tao1, raising the possibility of "bypass suppression" (in genetic terms) rather than a genuine rescue. Thus, it would seem critical to demonstrate restoration of SAC activity in Tao1-depleted cells that express murine Tao1 kinase at physiological levels. Unfortunately, a pool of HeLa cells expressing a LAP-tagged murine Tao1 could not be used in live-cell imaging because expression of LAP-murine Tao1 was too weak to be detected (data not shown). Expression of murine Tao1 could only be detected in fixed cells by immunofluorescence microscopy after treatment with a GFP-specific antibody (data not shown). Analysis of GFP-positive cells (expressing murine Tao1) by fluorescence activated cell sorting (FACS) revealed that the expression of LAP-murine Tao1 was nearly undetectable compared to HCT116 cells stably expressing histone H2B-GFP (Fig. 46).

Furthermore, we recognized that the designed siRNA oligonucleotide Tao1-NCB3 showed high sequence similarity to the murine mRNA and therefore would probably also target the mouse homologue. Thus, although it would seem critical to demonstrate restoration of SAC activity in Tao1-depleted cells by providing physiological levels of Tao1 kinase it is not possible to address this question further at this stage of research.

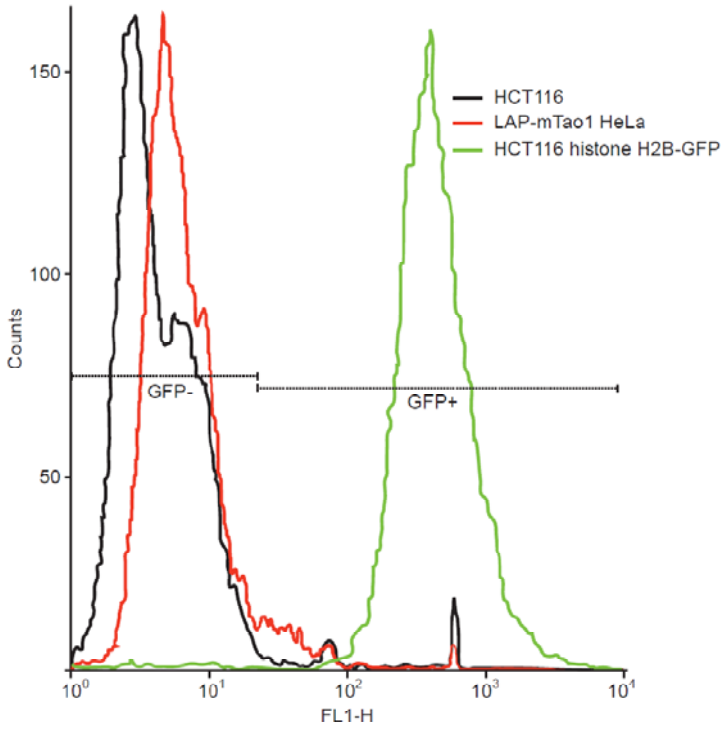



**Figure 46: Fluorescence activated cell sorting analysis of LAP-mTao1 HeLa cells.** *Histogram* illustrates the fluorescence intensity (FL1-H) of GFP of LAP-mTao1 HeLa cells (*red graph*) compared to parental HCT116 cells (negative control, *black graph*) and HCT116 cells stably expressing histone H2B-GFP (positive control, *green graph*). 20000 events were counted per cell line and defined (gated) as GFP non-expressing (GFP-) and GFP expressing (GFP+) cells. Only 6.4% of the LAP-mTao1 HeLa cell line was counted as GFP+ whereas 93.6% were counted as GFP-.

In the case of PICH, our results clearly demonstrate that overexpression of PICH protein restored SAC functionality in PICH-1 siRNA-treated cells (Fig. 31), and yet, physiological levels of LAP-mouse PICH provided no rescue (Fig. 44). The first evidence to explain this discrepancy emerged when we tested two PICH mutants for their ability to restore normal mitotic timing in PICH-1 siRNA-treated cells. In contrast to a PICH mutant with abolished ATPase activity (K128A, Leng et al., 2008) which had the ability to restore SAC activity in PICH-1 siRNA-treated cells, a mutant that cannot bind Plk1 due to a mutation within the Polo-box domain (T1063A) failed to restore proper mitotic timing (Fig. 47, in collaboration with Lily H.-C. Wang).

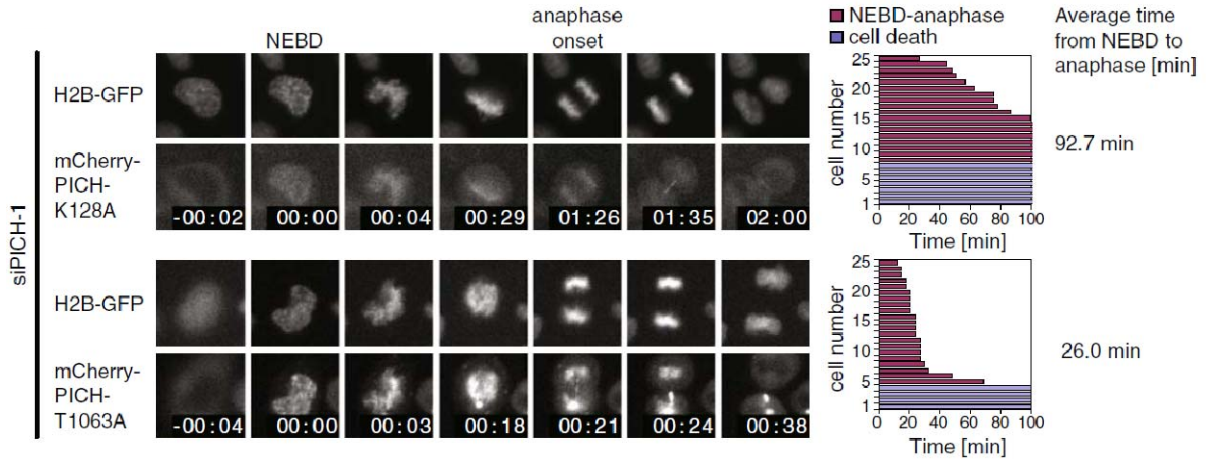



**Figure 47: Analysis of overexpressed PICH mutants for their ability to restore mitotic timing in PICH-1 siRNA-treated cells.** mCherry-PICH WT (shown in Fig. 31), mCherry-PICH-K128A (defective in ATPase activity), and mCherry-PICH-T1063A were co-transfected with PICH-1 siRNA oligonucleotide into H2B-GFP-expressing HeLa cells before these were analyzed by live-cell imaging (as described in the legend to Fig. 31). mCherry-positive cells were analyzed to determine the average time from NEBD to anaphase onset. *Left panels* show single-plane images selected from movies and time stamps indicate hours:minutes. *Right panels* show representative fate maps of 25 individual cells. The whole experiment being repeated independently three times, with *red columns* indicating the time span from NEBD to anaphase onset and *blue columns* indicating cells that died in interphase during imaging. Average times from NEBD to anaphase onset are indicated to the *right*.

These unexpected results could not be attributed to different expression levels of the rescuing constructs, as neither the transfection efficiencies nor the levels of overexpression achieved revealed significant differences (Fig. 48, in collaboration with L.H.-C. Wang).

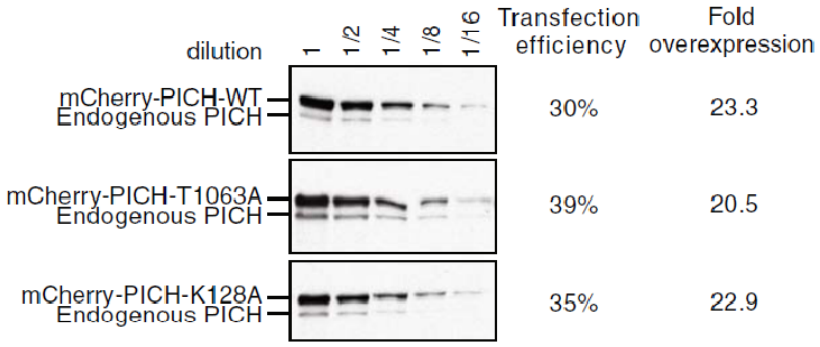



**Figure 48: Quantification of overexpression of mCherry-PICH WT, mCherry-PICH-K128A, and mCherry-PICH-T1063A in comparison to endogenous PICH.** HeLa cells were transfected with the indicated plasmids and total cell lysates were subjected to serial dilutions before Western blotting with anti-PICH antibody. Transfection efficiency (indicated to the *right*) was determined by immunofluorescence staining using anti-mCherry antibody, and fold overexpression was calculated, taking into account the dilution factor and the transfection efficiency. This experiment indicates that the mCherry-PICH constructs were similarly overexpressed (about 20–24-fold) when compared to endogenous PICH levels.

These results imply that restoration of SAC functionality in PICH-1 siRNA-treated cells might depend on the ability of the overexpressed PICH protein to sequester Plk1, rather than its ATPase activity. One possible interpretation of this result is that Mad2 protein can be utilized by the cell more efficiently to provide SAC activity upon depletion, sequestration or inhibition of Plk1. However, co-immunoprecipitation experiments revealed no change in MCC formation when Plk1 was depleted (Fig. 49) or inhibited by the small inhibitor TAL (data not shown).

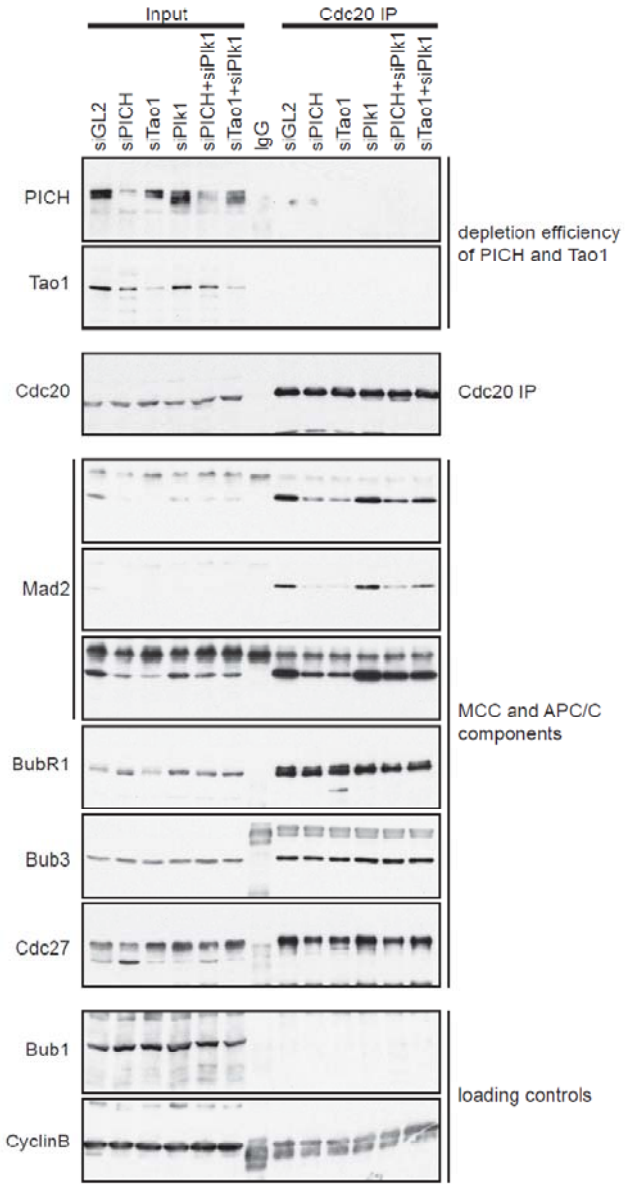



**Figure 49: Cdc20 co-immunoprecipitation of MCC components in cells treated with siPICH, siTao1 and siPlk1, respectively.** HeLa cells were depleted of PICH, Tao1 and Plk1, or co-depleted of PICH and Plk1 or Tao1 and Plk1, respectively. Cell lysates were incubated with protein G beads coupled to Cdc20 antibodies and blotted for MCC components. BubR1, Bub3 and Cdc27 are immuno-precipitated to same amounts, independent of siRNA treatment. Mad2 levels do not change between siGL2 and siPlk1 treated cells; however, less Mad2 is found within the MCC in PICH and Tao1 (and PICH+Plk1 and Tao1+Plk1, respectively) depleted cells due to the reduced input of Mad2 protein in these lysates.

Still, the above data clearly showed a mitotic rescue when Plk1 was sequestered by exogenous wild type or ATPase-deficient PICH protein. Therefore the observed restoration of SAC activity would represent a case of bypass suppression rather than a genuine rescue. To test this further, we performed live-cell imaging on HeLa cells stably expressing histone H2B-GFP and monitored mitotic progression under various treatments. Cells were depleted of PICH or Tao1, using PICH-1 or Tao1-NCB3 siRNA, respectively, and selected samples were subjected to additional depletion of Plk1 by siRNA or treated with TAL. While cells treated with PICH-1 siRNA alone showed no mitotic arrest in response to nocodazole (Fig. 30), co-depletion of PICH and Plk1 or inhibition of Plk1 by TAL resulted in mitotic delays of 110 and 155 min, respectively (Fig. 50a). These cells typically exited mitosis without chromosome segregation and often formed micronuclei (data not shown). As expected, single depletion of Plk1 or inhibition of Plk1 caused a prolonged prometaphase delay, mostly followed by apoptosis (Fig. 50, Liu and Erikson, 2003; Santamaria et al., 2007; Petronczki et al., 2008).

Similar results were obtained when the effect of Plk1 depletion or inhibition was analyzed on cells that were treated with Tao1-NCB3 siRNA oligonucleotides (Fig. 50b). In comparison to control cells which exhibited the expected mitotic timing, Tao1-NCB3 siRNA-treated cells performed mitosis in less than 20 min and without forming a metaphase plate. Simultaneous depletion of Plk1 by siRNA or its inhibition by TAL increased the timing to about 35 min, similar to the duration seen in control cells but far below the duration of the mitotic arrest seen after depletion of Plk1 alone. This suggests that Plk1 depletion or inhibition can marginally restore SAC activity in Tao1-NCB3 siRNA-treated cells, although Mad2 levels are low.

Based on these results we propose that residual Mad2 is critical for the restoration of SAC activity after Plk1 depletion/inhibition in PICH-1 or Tao1-NCB3 siRNA-treated cells. To demonstrate that residual Mad2 was critical for the restoration of SAC activity by Plk1 depletion/inhibition in PICH-1 or Tao1-NCB3 siRNA-treated cells, we also examined the consequences of combining Plk1 inhibition with direct siRNA-mediated depletion of Mad2. As expected, the addition of TAL could not restore normal mitotic timing in cells that were depleted of Mad2 (Fig. 50c). Therefore we argue that the absence of Plk1 activity cannot rescue SAC functionality in cells from which the bulk of Mad2 has been depleted. These results support the view that residual Mad2, as it persists after PICH-1 or Tao1-NCB3 siRNA treatment, is critical for the restoration of some SAC activity upon depletion or inhibition of Plk1.

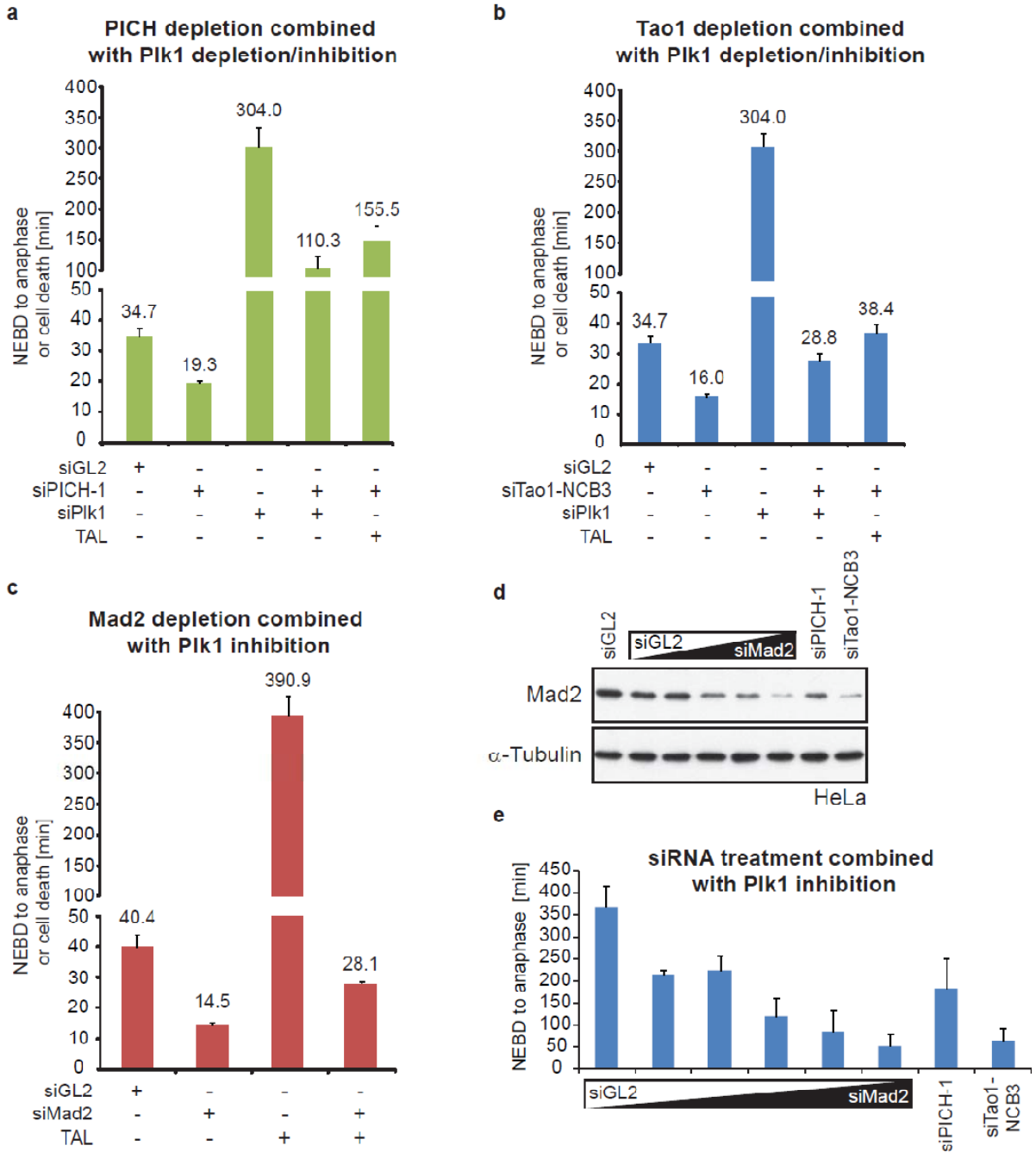



**Figure 50: Codepletion of PICH and Plk1 restores SAC activity. a–c** HeLa cells stably expressing H2B-GFP were subjected to single and double depletion (PICH, Tao1, Mad2, Plk1) or inhibition (Plk1) experiments, as described in each *panel*, and analyzed by time-lapse microscopy. *Histograms* illustrate the mitotic timing from NEBD to mitotic exit or cell death. *Error bars* represent standard error (n=40). **d** Western blot analysis of Mad2 abundance after transfection of HeLa cells with increasing concentrations of Mad2 siRNA duplex (0.06, 1.2, 3.2, 6.6, 20 pM) and PICH-1, Tao1-NCB3, and GL2 siRNA for comparison. α-Tubulin serves as loading control. **e** HeLa cells stably expressing H2B-GFP were transfected with increasing amounts of Mad2 siRNA (as described in d as well as PICH-1 and Tao1-NCB3 duplexes, prior to synchronization with thymidine and release into TAL-containing medium. Then, the mitotic timing from NEBD to anaphase onset was determined by live-cell imaging. *Histogram* illustrates the results from three independent experiments (n=20 for each experiment), and *error bars* represent standard error.

In a final set of experiments, different amounts of Mad2 siRNA oligonucleotides were used to reduce cellular Mad2 to levels within the range of those seen as a consequence of PICH or Tao1-NCB3 siRNA treatment. Live-cell imaging was used to analyze the mitotic timing in TAL-treated cells as a function of Mad2 levels. Strengthening our proposal about cellular Mad2-dependent SAC restoration, the mitotic timing (NEBD to anaphase onset) observed in response to Plk1 inhibition showed a strong correlation with Mad2 protein levels (Fig. 50d). Furthermore, Tao1-NCB3 siRNA resulted in a more severe depletion of Mad2 than PICH-1 siRNA and, concomitantly, a more pronounced advancement of mitotic timing. We therefore propose that restoration of SAC activity by depletion or inhibition of Plk1 depends on residual levels of Mad2.

The mechanism how depletion or inhibition of Plk1 enhances the functionality of Mad2 remains to be investigated; but these observations point to the conclusion that, under physiological circumstances, Plk1 antagonizes the SAC signaling function of Mad2.

### 2.9. Oligonucleotide sequence alignments

Off-target effects occur if a siRNA oligonucleotide designed to knockdown a specific mRNA, randomly targets other mRNA transcripts to knockdown expression of these non-targeted genes. The above data demonstrated that Mad2 levels were frequently reduced by the usage of some siRNA oligonucleotides originally designed to target PICH or Tao1. Considering that the alignment of the Mad2 mRNA sequence with the sequences of the various PICH- or Tao1-directed oligonucleotides revealed no evidence for extensive complementarities (Fig. 51), the Mad2 transcript is unlikely to be the primary target of these siRNA duplexes. Instead, we presume that the PICH-1, PICH-2, PICH-CC and Tao1-NCB3 duplexes target other unidentified transcript(s), which then affect Mad2 mRNA levels through indirect mechanisms. Furthermore, we cannot exclude the possibility that other cellular processes, such as microRNA-based gene regulation, are affected by the transfection of specific siRNA oligonucleotides, which then alter the expression of Mad2.


**Figure 51: Alignment of PICH and Tao1 siRNA sequences with Mad2 mRNA.** The alignments were performed using the global EMBOSS pairwise Alignment Algorithm (http://www.ebi.ac.uk/Tools/emboss/align) with a gap open of 50.0 and the Mad2 RNA accession number NM_002358.3. The most extensive match observed for each oligonucleotide is shown. Alignments colored in *red* or *green* refer to siRNA duplexes that do or do not lower Mad2 transcripts, respectively.

## 2.10. Conclusion Part 2

The conclusions emerging from this study question the previously reported requirements of PICH (Baumann et al., 2007) and Tao1 (Draviam et al., 2007) for SAC signaling. In contrast we conclude that the previously published siRNA-based observations of PICH and Tao1 reflect an off-target effect on the checkpoint protein Mad2. At present, the primary target of these siRNAs remains unclear and the precise physiological function of both proteins awaits further studies.

In the case of PICH we clearly believe in a possible role in chromosome structure alteration and/or segregation based on its unique localization to centromeres, KTs and ultrafine DNA bridges and its physical interaction with Plk1 (Kurasawa and Yu-Lee, 2010; Baumann et al., 2007; Santamaria et al., 2007). The results on Tao1, however, differ from those reported in the original study with regard to the effect of siRNA on Mad2 and the advancement of mitotic timing (Draviam et al., 2007). In contrast to the data published by Draviam and coworkers a recent publication supports our view that depletion of Tao1 by the Tao1-NCB3 siRNA

oligonucleotide reflects an off-target effect on Mad2 (Westhorpe et al., 2010). However, Tao1 kinase has been found to play a role in the regulation of MTs and might be considered to contribute to mitotic events by this function (Timm et al., 2003; Johne et al., 2008).

In this study, we have discovered that the apparent "rescue" of mitotic timing (caused by the depletion of PICH with the previously published oligonucleotide siPICH-1) depended on the ability of overexpressed PICH protein to sequester the mitotic kinase Plk1. Thus, it is intriguing to speculate that Plk1 normally reduces the strength of the Mad2-dependent inhibitory SAC signal or in other words, Plk1 depletion supports SAC activation even at low Mad2 protein levels. In future studies, it will clearly be interesting to explore the molecular mechanism(s) by which Plk1 affects SAC functionality.

## 3. Expression, purification and crystallization of PICH protein

The three-dimensional structure of a protein provides important information about its architecture and is often essential to understand functionality. Among others, X-ray crystallography is a central method in determining the structure of proteins at atomic resolution. Since many materials, including biological macromolecules can form crystals, biochemists and X-ray crystallographers use this method to solve the structure of proteins. One crucial step that limits structural studies is the production of the respective protein in high and pure amounts needed for crystallization. Considering that the role of PICH during mitosis remained unclear from the above studies we tried to gain information about the function of PICH by solving its crystal structure. These experiments were performed in collaboration with the laboratory of Karl-Peter Hopfner at the Gene Center Munich.

### 3.1. Expression and purification of PICH from insect cells

A crucial step in X-ray crystallography is the expression and purification of the respective protein. In foregoing work, Sebastian Fenn (group of Karl-Peter Hopfner) designed and expressed several C-terminally truncated constructs, as well as full length PICH protein in *Escherichia coli.* Unfortunately, bacterial expression of all designed constructs either yielded no or insoluble protein (data not shown). Re-folding of insolubly expressed protein did not lead to correctly folded and soluble protein either. Thus, a baculovirus based expression system was used to express N-terminally His-tagged PICH recombinantly in insect cells. The baculovirus was generated in Sf21 cells and used for protein expression in H5 cells (see Materials and Methods). Ni-NTA column purification indicated a small amount of soluble PICH full length protein (Fig. 52a). Small amounts of PICH full length protein were further purified by an ion exchange column and gel filtration (Fig. 52b). Shorter constructs designed from the N-terminus, encompassing its ATPase domain were either not expressed or insoluble (data not shown).

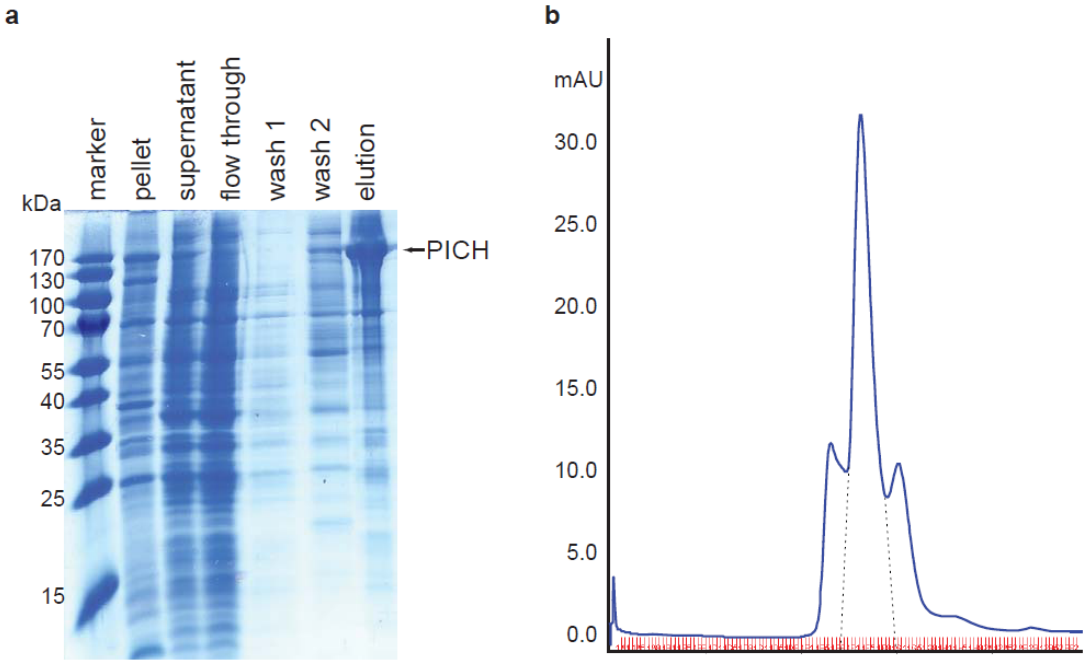



**Figure 52: Ni-NTA pull-down of His-tagged PICH full length protein.** His-tagged PICH protein was expressed in one liter shaking culture H5 cells and harvested after 72 h of baculovirus infection. **a** The protein was pre-purified via a Ni-NTA column and analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. **b** After ion exchange column purification His-PICH full length was purified by gel filtration. Peak fractions (*dashed line*) were pooled and concentrated before crystal screen set-ups.

The expression of PICH was unfortunately very low in insect cells. Starting with 1 l shaking insect cell culture (1x$10^6$ cells/ml) we obtained approximately 300 µl of full length PICH with a concentration of ~4.5 µg/µl after the complete purification protocol. The purified protein was used for crystal screens with a final concentration of PICH full length protein of 2.6 µg/µl and 4.4 µg/µl. No crystals were obtained in four different commercially available 96-well format sitting-drop screens at 16°C.

### 3.2. Recombinant full length PICH binds to double stranded DNA

Purified His-tagged PICH full length protein was analyzed by electrophoretic mobility shift assay (EMSA) for its capacity to bind to double stranded DNA (dsDNA), single stranded DNA (ssDNA) or DNA aligned to form Holiday Junctions (HJ, Fig. 53). All DNAs were kindly provided by Julia J. Griese.

Although PICH binds to ssDNA the protein appears to have a preference for binding to dsDNA. No additional effect was seen when Holiday Junctions were incorporated into the DNA. Other DNA sequences tested (50mer dsDNA, 50mer ssDNA, or loop-structure) did not reveal any other binding preference (data not shown).

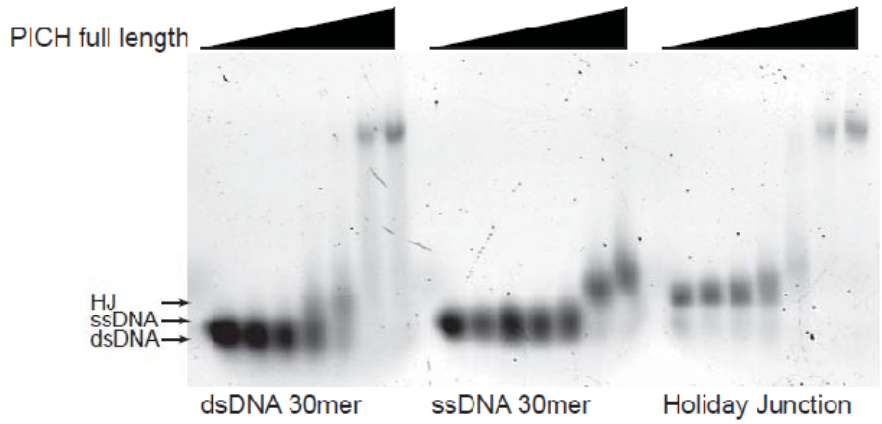



**Figure 53: PICH full length protein prefers binding to dsDNA.** Increasing amounts of His-tagged PICH full length protein were incubated with 50 nM of dsDNA (random sequence), ssDNA (poly dT), or DNA annealed to form a Holiday Junction and analyzed by EMSA.

If PICH significantly prefers one specific DNA structure over another or if sequence specificity (e.g. α-satellite repeats) plays a major role in PICH binding to DNA needs to be evaluated in future experiments.

### 3.3. Conclusion Part 3

The three-dimensional structure of a protein often provides key insights into possible functions and potential biochemical mechanisms the respective protein might be involved in. Thus, by solving the structure of PICH by X-ray crystallography we expected to gain information about the domain architecture of PICH and to link the unique localization of PICH to centromeres, KTs and ultra-fine DNA threads to its function. Unfortunately, we faced many problems while expressing the approximately 140 kDa protein. Full length protein or shorter N-terminal constructs that were expressed in *Escherichia coli* were insoluble or not produced. The baculovirus expression system in insect cells yielded small amounts of full length PICH; however, no crystals were obtained in four different crystal screens (in total 384 different conditions). N-terminal fragments were either not expressed or found in very small amounts as insoluble protein.

Conclusions emerging from the last series of experiments show that recombinant PICH binds to several DNA structures. Hence, we are confident that PICH plays an important role during mitosis by binding to DNA and possibly altering chromosome structure and/or function in chromosome segregation.

# V. DISCUSSION

PICH has been described as a SAC component whose localization to KTs, centromeres and chromosome arms is controlled by the mitotic kinase Plk1 (Baumann, 2007; Santamaria et al., 2007; Leng et al., 2008). However, other interaction partners and the mechanism of the initial recruitment of PICH to the KT/centromere remained unknown. This lack of knowledge prompted us to investigate possible interaction partners of PICH and our findings led us to hypothesize that the chromosomal passenger protein Aurora B is essential for KT/centromere recruitment as well as chromosome arm localization of the ATPase PICH.

### 1. PICH is recruited by Aurora B and the Ndc80 complex

Aurora B is the catalytically active subunit of the chromosomal passenger complex (CPC), which further comprises INCENP, Survivin, and Borealin (Musacchio and Salmon, 2007; Ruchaud et al., 2007a). Our data indicate that PICH is regulated by the CPC. In particular, PICH is absent from the KT/centromere in CPC-depleted cells but is not influenced by Aurora B kinase inhibition (see also Klein, 2008). To our knowledge, the requirement for the CPC in recruiting KT/centromere proteins has so far always been assigned to the enzymatic activity of Aurora B (Ditchfield et al., 2003; Hauf et al., 2003; Emanuele et al., 2008). Thus, with regard to the CPC and Aurora B kinase activity, PICH recruitment is unique among tested candidates and published results. As PICH localization could not be rescued with the ternary $INCENP^{1-58}$-Survivin-Borealin subcomplex or the Aurora B binding deficient mutant $INCENP^{F825A/F837A}$, the information required for PICH KT/centromere binding must be present within the Aurora B protein. The established rescue assay should now allow for the identification of the corresponding region of Aurora B.

In addition to the Aurora B-dependent recruitment, we found the Ndc80 complex (comprising human Hec1, Nuf2, Spc24 and Spc25) to be required for the KT/centromere localization of PICH. Specifically, PICH is absent from the KT/centromere in cells depleted of Hec1 or Nuf2. The Ndc80 complex has been reported previously to be required for microtubule (MT) attachment, chromosome congression and KT assembly (Martin-Lluesma et al., 2002; DeLuca et al., 2003; Maiato et al., 2004; DeLuca et al., 2005). Our data indicate that PICH mislocalization upon Ndc80 depletion is unlikely to solely depend on the disruption of MT-KT interactions. We thus propose two possible scenarios which are not mutually exclusive. First, the CPC and Ndc80 complex are required to stably recruit PICH to the KT/centromere,

suggesting a cooperative mechanism. In turn, we speculate that the Ndc80 complex is initially needed to recruit PICH, possibly also through stable MT-KT interactions. Once recruited, Aurora B protein, but remarkably not its kinase activity, is needed to maintain PICH localization. We also cannot exclude the possibility of a yet unidentified interaction partner mediating the connection between Aurora B, Hec1/Nuf2, and PICH. Future studies will hopefully identify the mechanism behind the CPC/Ndc80-dependent recruitment of PICH to the KT/centromere.

**2. PICH – a mitotic target of Plk1 and Aurora B**

The CPC, Plk1 and PICH have been demonstrated to regulate chromosome arm cohesion. According to the current model, arm-associated cohesin is removed by Plk1-mediated phosphorylation of the SA2 subunit of the cohesin complex (Sumara et al., 2002; Gimenez-Abian et al., 2004; Hauf et al., 2005), while centromere-associated cohesin holds sister chromatids together until the inactivation of the SAC results in separase activation and cohesin cleavage (Waizenegger et al., 2000). PICH in turn, is essential to localize Plk1 to the chromosome arms (Santamaria et al., 2007; Leng et al., 2008) and cells depleted of PICH show closed chromosome arms (Leng et al., 2008). The requirement for the CPC in the resolution of arm cohesion has been reported previously but the molecular basis has remained elusive (Losada et al., 2002; Gimenez-Abian et al., 2004). In this study, we provide evidence that the CPC is required for PICH to localize to chromosome arms, which in turn recruits Plk1 to release cohesin via the phosphorylation of SA2. To our surprise, double depletion of Aurora B and Plk1 mislocalized PICH from KTs/centromeres, whereas simultaneous inhibition of both kinases resulted in KT/centromere, but strikingly not chromosome arm localization. Therefore, it seems attractive to speculate that the removal of cohesin from chromosome arms is mediated through the concerted actions of Aurora B, Plk1 and PICH. Hence, we envision the following working model (Fig 54): During prophase Aurora B kinase activity is required to initially recruit PICH to chromosome arms. The Snf2 family ATPase PICH would then be ideally suited to alter chromosomes and chromatin structure by the ATP-dependent translocation on DNA. Unfortunately, the mechanism behind the dependency of PICH on Aurora B remains unclear. Although we were able to show that PICH is phosphorylated by Aurora B *in vitro* and *in vivo*, we cannot exclude the possibility that an as yet unidentified protein is required for the connection between Aurora B and PICH.

As the cell undergoes mitosis, increasing Cdk1 activity allows phosphorylation of T1063 of PICH, which primes PICH for Plk1 binding and the recruitment of Plk1 to chromosome arms

(Leng et al., 2008). Plk1 in turn is now perfectly suited to resolve chromosome arm cohesion, finally releasing PICH from chromosome arms, possibly by direct phosphorylation. This model plausibly combines the requirement for Aurora B kinase activity to localize PICH to chromosome arms and the fact that Plk1 kinase activity is a prerequisite to remove PICH from chromosome arms.

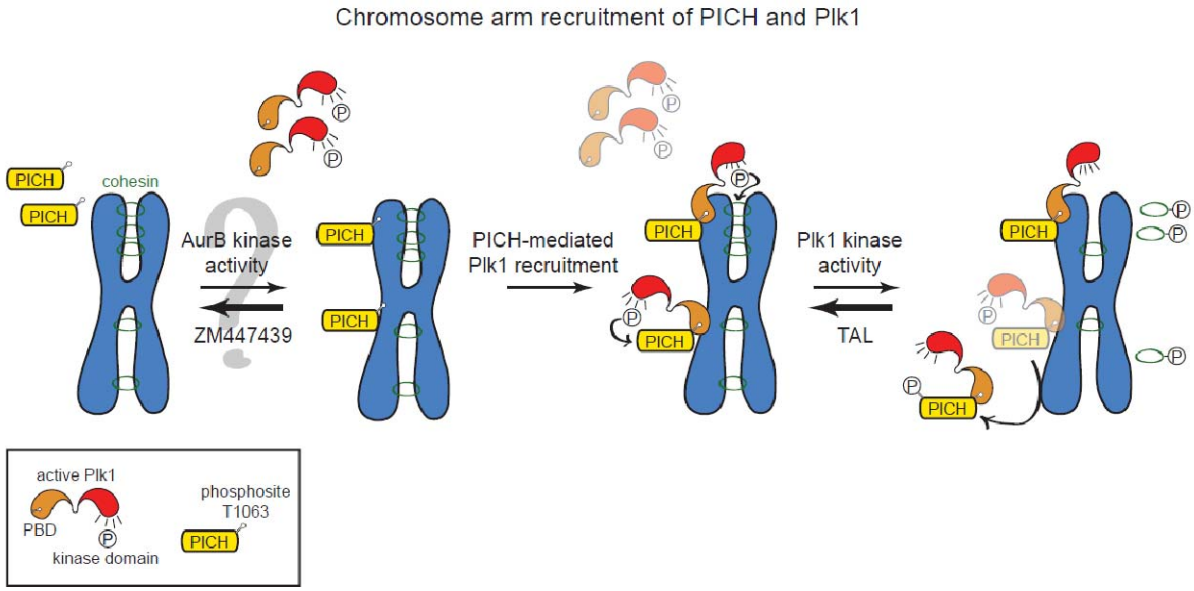



**Figure 54: Working model for the recruitment of Plk1 to chromosome arms.** In a first step, the Aurora B kinase is required to locate PICH to chromosome arms. This step highly depends on the kinase activity of Aurora B as inhibition by ZM447439 mislocalizes PICH from chromosome arms after TAL treatment. It seems plausible that the Snf2 protein PICH alters DNA at this location dependent on its ATPase activity. Second, PICH, which has been phosphorylated at its "priming" site T1063 binds Plk1, which in turn is now perfectly suited to phosphorylate its target proteins (such as the cohesin ring complex) as well as PICH. The Plk1-mediated phosphorylation of PICH allows the PICH-Plk1 complex to finally leave the chromosome arms. As a consequence, inhibition of Plk1 kinase activity by TAL treatment causes the accumulation of PICH and Plk1 at chromosome arms.

Note that this model only provides an explanation for the chromosome arm recruitment of PICH and Plk1. KT/centromere localization of PICH, however, seems to be regulated by a different mechanism and the fact that PICH is not removed from the KTs/centromeres by Plk1 remains to be understood. Nevertheless, the role of Plk1in controlling PICH localization during mitosis is highly reminiscent of the function of Plk1 in removing cohesins from chromatid arms (Losada et al., 2002; Sumara et al., 2002; Hauf et al., 2005), whereas centromeric cohesin is protected by the action of Sgo1 and the phosphatase PP2A (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006). Although the possibility of a "centromeric-PICH protection pathway" cannot be neglected, data identifying a "PICH-protector" are

missing. Thus, further studies are required to dissect the regulatory pathways that control PICH at the KT/centromere region and chromosome arms.

### 3. The CPC and the spindle checkpoint

Kinetochore tension arises because of the pulling forces exerted by spindle microtubules (MTs) and the resistance offered by the physically tethered sister chromatids. The SAC is supposed to sense improper MT attachments and/or lack of tension; however, the primary signal that activates the SAC has remained a matter of debate because of the interdependency of tension and attachment (Pinsky and Biggins, 2005). In short, the question remains whether the spindle checkpoint directly senses the lack of tension or whether it indirectly does so by reducing KT-MT attachments through the activity of Aurora B (Maresca and Salmon, 2010).
Interestingly, cells depleted of the CPC arrest in mitosis in response to nocodazole treatment (lack of MT attachments and thus tension) but exit mitosis when cells are treated with taxol (established MT attachments but no tension generated). The CPC is therefore considered to represent the tension sensing part of the SAC (Tanaka et al., 2002; Musacchio and Salmon, 2007; Ruchaud et al., 2007b). Furthermore, the protein PICH had originally been proposed to sense tension due to its unique localization to KTs and centromeres as well as ultra-fine DNA bridges which often connect sister KTs in anaphase. Although the detailed mechanism remained unclear, PICH was also suggested to regulate the SAC via the KT recruitment of Mad2 (Baumann et al., 2007). As the CPC is required for KT/centromere targeting of PICH we tested if the SAC override seen in CPC-depleted cells treated with taxol but not nocodazole might be assigned to PICH KT/centromere localization. Upon CPC depletion, we observed PICH to be absent from the KT/centromere in both conditions (nocodazole and taxol). Remarkably and most interestingly, the checkpoint protein Mad2 that similarly depends on the CPC was re-directed to the KTs in Aurora B-depleted cells under conditions of nocodazole treatment. Two important conclusions emerge from this experiment. First, the striking difference in Mad2 KT localization provides a plausible explanation for the different response of CPC-depleted cells to the two MT poisons nocodazole and taxol. And second, the result indicates that Mad2 recruitment to the KT is independent of PICH KT/centromere localization, contrary to expectation (Baumann, 2007). This result does indeed have important implications on the proposed function of PICH to uphold conditions required for the establishment of inhibitory Mad2 at the KT.

**4. Re-evaluation of the role of PICH in the spindle checkpoint**

The early model envisioned a role for PICH in the regulation of centromere-associated DNA and in SAC signaling during mitosis. How PICH might uphold conditions permissive for the establishment of inhibitory Mad2 at the KTs and thereby act as a major regulator of the SAC remained unclear. Our studies have shown that depletion of PICH by previously published siRNA oligonucleotides results in an off-target effect on the checkpoint protein Mad2. Therefore, we question the previously reported requirement for PICH in the SAC (Baumann et al., 2007). At present, the precise physiological function of PICH awaits further investigation; but in analogy to the purported roles of other Snf2 family members, which utilize ATP hydrolysis to either displace proteins from chromatin, translocate on double stranded DNA or generate superhelical torsion (Becker and Horz, 2002; Svejstrup, 2003; Beerens et al., 2005; Lia et al., 2006), it seems plausible that PICH may induce changes in DNA topology or remodel centromeric chromatin during mitosis. This view is supported by the unique localization of PICH to centromeres, KTs, and ultra-fine DNA bridges and its physiological interaction with Plk1.

In principle, an off-target effect could also be attributed to those siRNA duplexes that do not lower Mad2 expression. In particular, one could argue that PICH plays a physiological role in controlling Mad2 expression and that the failure of some duplexes to affect Mad2 levels (and hence checkpoint functionality) might reflect off-target effects interfering with this (hypothetical) regulation. This line of argument also includes off-target effects caused by PICH siRNA oligonucleotides and leaves the target protein unknown. A genetic PICH knockout will be required to formally exclude this rather unlikely alternative interpretation.

**5. Off-target effects and the connection to Plk1 function in SAC functionality**

In 2006, the Nobel Prize in Physiology or Medicine was awarded for the discovery of RNA interference (RNAi, Fire et al., 1998). This mechanism involves double stranded RNA (dsRNA) that is cleaved into fragments of 21 nucleotides (called small interfering RNAs, siRNAs), which trigger suppression of gene activity in a sequence-dependent manner (Elbashir et al., 2001). Their discovery revealed a new mechanism for gene regulation, and the biochemical machinery involved plays a key role in many essential cellular processes (Hannon, 2002; Agrawal et al., 2003; Denli and Hannon, 2003). Today, siRNA is used as a powerful tool to experimentally suppress specific genes and look for the resulting phenotypic effect. As a consequence, essentially any gene in an organism can now be studied

functionally. Unfortunately, RNAi is not without complications. The biggest concern questions the specificity of siRNAs which usually is related to their sequence. For example, sequence identity of 11 contiguous nucleotides to a siRNA caused direct silencing of non-target genes in experiments conducted on the specificity of siRNA in cultured human cells (Jackson et al., 2003; Scacheri et al., 2004). This phenomenon is known as "off-target effect" and difficult to detect. Of course, lack of specificity resulting in knockdown of unknown or unintended genes has considerable negative implications for functional genomics and the evaluation of the function of a certain gene and its expression product. In turn, the examination of cellular phenotypes based on siRNA studies can be misleading due to a possible off-target effect, which often is not explicitly controlled for on a routine basis.

Currently, BLAST similarity searches (Altschul and Lipman, 1990) against sequence databases are used to identify potential off-target genes and to improve the likelihood that only the intended single gene is targeted (Elbashir et al., 2002).

In general, rescue experiments are accepted as "gold standard" for assessing siRNA specificity. However, our study illustrates that even rescue experiments must be interpreted with caution, unless rescuing proteins are re-expressed at physiological levels. In this study, we were able to show that an apparent "rescue" depended on the ability of the rescuing protein to sequester Plk1. Specifically, in contrast to the Plk1-binding mutant (PICH T1063A), overexpression of wild type and an ATPase-deficient mutant of PICH (K128A) did rescue mitotic timing in cells depleted of PICH by siRNA. Hence, we suggest the following explanation (Fig. 55). Mitotic timing (NEBD to anaphase onset) is highly dependent on cellular Mad2 levels, as even partial reduction of Mad2 cause a prominent phenotype. Under normal conditions, the expression level of Mad2 is regulated in a way that the SAC is turned off once all KT are attached to spindle MTs. Reduction of Mad2 by PICH, Tao1 or Mad2 siRNA logically results in SAC abrogation. Interestingly, co-depletion of PICH, Tao1 or Mad2 together with Plk1 restored SAC functionality, dependent on the residual amounts of cellular Mad2. Based on this result we suppose that Plk1 normally reduces the strength of the Mad2-dependent inhibitory SAC signal. At present we do not have any evidence that Plk1 is directly involved in the repression of Mad2 utilization at the KTs. It remains possible that Plk1 acts further downstream, for example at the level of the APC/C and it will be of great interest to investigate molecular details by which Plk1 operates in SAC functionality. The rescue experiments described above underline the importance of Plk1 for SAC signaling. Sequestered Plk1 was not able to perform its function in Mad2 utilization comparable to the

situation of Plk1 depletion or inhibition. As a consequence, the spindle checkpoint depends on Mad2 protein levels and Plk1 accessibility (Fig. 55).

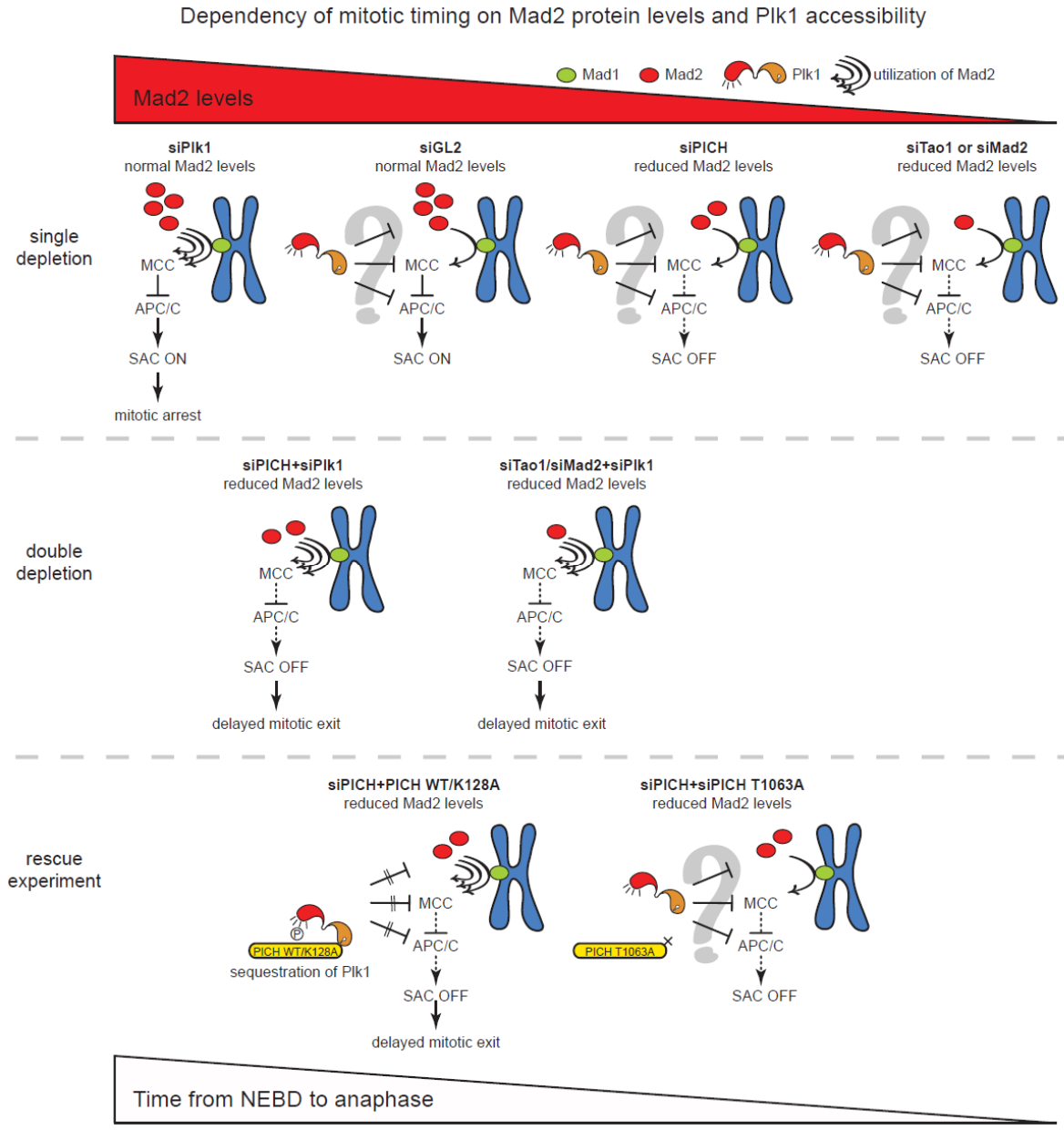



**Figure 55: Working model describing the influence of Mad2 levels and the accessibility of Plk1 on mitotic timing.** Depletion, inhibition or sequestration of Plk1 restored SAC activity in cells with reduced Mad2 protein levels.

Expression of the mouse homologue of PICH (Ercc6l) to near-physiological levels was not able to restore mitotic timing after PICH depletion. Thus, we conclude that either mouse PICH is not able to provide SAC functionality in human cells or that the used tag (LAP-tag) interfered with protein functionality. A third explanation would be that PICH is not the only target of PICH-1 siRNA, which is indeed the case as the checkpoint protein Mad2 is affected

in mRNA and protein levels. Given the fact that PICH and Ercc6l share high sequence similarity, we argue for the last explanation, although we cannot completely exclude a possible constraining role of the LAP-tag. The rescue of mitotic timing by expression of mouse Mad2 under the physiological promoter in PICH-depleted cells supports this assumption. We further argue that under conditions of PICH depletion and expression of Ercc6l to physiological levels, a "false rescue" will not be detected, due to the missing sequestration of Plk1. This interesting case of bypass suppression further suggests that Plk1 normally reduces the strength of the Mad2-dependent inhibitory SAC signal. In future studies, it will clearly be interesting to explore the molecular mechanism(s) by which Plk1 affects SAC functionality.

**6. Re-evaluation of the role of Tao1 in the spindle checkpoint**

Mad2 is an essential protein to delay anaphase onset under all conditions known by activating the spindle checkpoint until bipolar attachment of spindle MTs to KTs has been achieved (Hardwick et al., 1999; Shannon et al., 2002). The Tao1 kinase had been identified as a new (apparent) spindle checkpoint component and siRNA-based depletion of Tao1 resulted in the mislocalization of Mad2 (but not Mad1) from the KTs and failure of SAC signaling (Draviam et al., 2007). This result is highly reminiscent of what has been observed for PICH. Thus, we investigated a possible role for Tao1 and PICH in regulating the SAC. To our surprise, some of our results on Tao1 differ from those reported in the original study (Draviam et al., 2007). Notably, our observations strongly suggest that the SAC override induced by the published siRNA oligonucleotide is due to its ability to repress Mad2, not Tao1. Other siRNA oligonucleotides (Tao1-NCB1 and Tao1-NCB2) used by Draviam and coworkers did not result in a mitotic phenotype and depletion of Tao1 by newly designed siRNA oligonucleotides (this study) had no effect on Mad2 protein and mRNA levels as well as mitotic timing or nocodazole arrest. In addition, an independent study demonstrated recently that the oligonucleotide Tao1-NCB3 results in off-target repression of Mad2 (Westhorpe et al., 2010). In turn, we find it difficult to escape the conclusion that the previously siRNA-based implication of Tao1 in the SAC reflects an off-target effect on Mad2, comparable to the observations for PICH.

We emphasize that Draviam and coworkers reported effective rescue of Tao1 depletion by active but not inactive kinase (D151A mutation). This raises an important question. If Tao1 siRNA actively targets Mad2 (directly or indirectly), how can the overexpression of Tao1 rescue the loss of Mad2? First, the detailed characterization of siTao1-NCB3 was performed

using time-lapse microscopy, the reported rescue experiment, however, relied on fixed cell assays. Interpreting fixed cell experiments can be problematic when assessing perturbations in the cell cycle (Rieder and Maiato, 2004), as for example a failure in SAC arrest could conceivably be a failure of cells to enter mitosis. Second, overexpression experiments must be interpreted with caution, as we have in fact discovered that the apparent "rescue" of a siRNA-mediated phenotype depended on the ability of the overexpressed PICH protein to sequester the mitotic kinase Plk1 (Fig. 54). This might not be the case for Tao1, but overexpression of Tao1 might have inhibited mitotic entry. Tao1 has been implicated in p38 regulation, which in turn functions in regulating the G2 to M transition, thus providing a possible link between Tao1 and mitotic entry (Hutchison et al., 1998; Yustein et al., 2003; Cha et al., 2007; Raman et al., 2007). Note that the above mentioned points display possibilities trying to explain the discrepancy between the published data (Draviam et al., 2007) and our study. In the future, rescue experiments in which Tao1 kinase is expressed at physiological levels are required to definitively validate or exclude the role for Tao1 in the SAC. In any case, considering the role of Tao1 in the regulation of microtubules (Timm et al., 2003; Johne et al., 2008), an important contribution to mitotic events is to be expected.

**7. Mad2 – an unintentional target of siRNA experiments?**

The DNA-dependent ATPase PICH and the microtubule affinity-regulating kinase kinase Tao1 have initially been identified as important regulators of mitotic progression (Baumann et al., 2007; Draviam et al., 2007). Unfortunately, based on our study, we find it difficult to escape the conclusion that the previous siRNA-based implications for both proteins in the SAC reflect an off-target effect on Mad2. Considering that the alignment of the Mad2 mRNA sequence with the sequences of the various PICH- or Tao1-directed oligonucleotides revealed no evidence for extensive complementarities (Fig. 51 and data not shown), the Mad2 transcript is unlikely to be the primary target of these siRNA duplexes. Instead, we presume that the PICH-1, PICH-2, PICH-CC, and Tao1-NCB3 duplexes target other transcripts, as yet unidentified, which then affect Mad2 expression or stability by indirect mechanism(s). Future studies including microarray experiments and/or deep sequencing will hopefully shed light on the target range of these siRNA oligonucleotides. In turn, the identification of critical targets may contribute to a better understanding of the mechanisms that regulate Mad2 expression.

Why Mad2 appears to be such a sensitive, albeit unintentional target of siRNA experiments remains elusive. We consider at least three plausible explanations, which are not mutually exclusive. First, although many proteins (including SAC components) provide functionality

even at reduced levels, exact levels of Mad2 seem to be critical for its flawless function (Michel et al., 2001; Hernando et al., 2004). Therefore, even partial reductions in Mad2 levels are expected to produce pronounced phenotypes. Second, the loss of SAC activity that accompanies the lowering of Mad2 levels is relatively easy to score in cellular assays. Thus, a general prediction emerging from the present study is that off-target effects will be identified more readily if they converge onto proteins that are critically involved in cellular processes that can easily be monitored. Third, Mad2 plays a central role in SAC signaling and so its expression is likely to be regulated by multiple mechanisms. For example, Mad2 transcription is regulated by the retinoblastoma and E2F pathway (Hernando et al., 2004). This provides a broad range of transcripts, upstream factors and pathways that could potentially be modulated by small RNAs, including siRNAs that might function as microRNAs, and thus influence Mad2 protein levels. Hence, if the ability of several siRNA oligonucleotides to repress Mad2 is not simply a direct off-target effect, then understanding this process may provide insights into an important pathway regulating Mad2 expression.

In summary, our study illustrates that, although controlled by several siRNA oligonucleotides directed against a specific gene and/or positive rescue experiments, RNAi can be misleading with regards to the examination of the functionality of a single gene. However, it remains of much interest to elucidate the regulatory network behind Mad2 expression and regulation before and after transfection of several siRNA oligonucleotides, and the role of Plk1 in SAC signaling. In the case of PICH, understanding its unique localization and associated mitotic function(s) awaits further studies on possible interaction partners, such as Aurora B and other KT proteins and kinases, enzymatic studies on its predicted ATPase, as well as structural insights into different predicted domains.

# VI. MATERIALS AND METHODS

### 1. Cell culture and synchronization

HeLa, U2OS, 293T and HeLa cells stably expressing H2B-GFP were grown under standard conditions in DMEM (Invitrogen) supplemented with 10% FBS and penicillin and streptomycin (100 U/ml and 100 mg/ml, respectively). Cell cycle synchronizations were performed by a single thymidine (2 mM) block 10-12 h post-transfection with siRNA duplexes. For microscopic analysis of mitotic cell cycle stages, cells were released from thymidine for 12 h before fixation. Mitotic cell cycle arrest in prometaphase was induced by nocodazole (0.2 μg/ml) or taxol (1 μg/ml) treatment for 16 h and mitotic cells were isolated by mitotic shake-off. The stable 293T TREX-shPICH cell lines were prepared under selective conditions using blasticidine (5 μg/ml) and puromycin (1 μg/ml) and grown under standard conditions. Stable clones were picked and analyzed by Western blotting and immunofluorescence microscopy before and after tetracycline induction. HeLa cells (pools) containing BACs expressing C-terminally LAP-tagged mouse PICH (Ercc6L, BAC-ID RP24-326A8), mouse Mad2L1 (BAC-ID RP23-84G11) or mouse Tao1 (BAC-ID RP23-315G8) were produced as described previously under 400 μg/ml G418 selection (Hutchins et al., 2010; Poser et al., 2008). In brief, bacterial artificial chromosomes were obtained from the BACPAC Resources Center (http://bacpac.chori.org), the LAP (EGFP-IRES-Neo)-cassette was amplified by PCR using primers that carry homology to the C-terminus of Ercc6L, Mad2L1 or Tao1, respectively, prior to transfection of the modified BAC. Sf21 cells (used for baculovirus amplification) were grown in suspension culture flasks in Sf-900 II serum-free medium (Invitrogen) supplemented with gentamycin (10 μg/ml) at 27.5°C. H5 cells (used for baculovirus-induced protein expression) were grown in suspension culture flasks in Express Five serum-free medium (Invitrogen) supplemented with gentamycin (10 μg/ml) and L-glutamine (1.5 mg/ml).

### 2. Transient transfection, siRNA and plasmid construction

Plasmid and siRNA transfections in human cell lines were performed using TransIT-LT1 transfection reagent (Mirus) and Oligofectamine reagent (Invitrogen), respectively, according to the manufacturer's instructions. Plasmid transfection in Sf21 cells was performed using FuGene (Roche) according to the manufacturer's instructions. Sequences of siRNA duplexes used in this study are listed in the Appendix.

To clone PICH constructs for expression in HeLa cells, PICH cDNA was amplified by PCR using pEGFP-C1-PICH (WT and T1063A) as a template (Baumann et al., 2007) and primers M3863 and M3864. The Walker A mutant (K128A) was generated by site-directed mutagenesis using M3821 and M3822. For rescue experiments, mCherry-tagged PICH constructs were used. The cDNA for mCherry was PCR amplified from pRSET-B-mCherry (a kind gift from Roger Y. Tsien), using the M3914 and M3915 primers. The mCherry construct was then subcloned into KpnI and EcoRV sites of the pcDNA4/TO vector. Both wild type and mutant PICH cDNAs were subcloned into the pcDNA4/TO-mCherry vector. To make constructs resistant against PICH-1 siRNA, the above constructs were then subjected to site-directed mutagenesis using the M3344 and M3345 primers. For construction of the shPICH plasmid, the oligonucleotides M3666 and M3667 were annealed in annealing buffer (200 mM Tris pH7.5, 100 mM $MgCl_2$ and 500 mM NaCl) for 1 h at 37 °C and cloned into pTER+ vector using BglII and HindIII restriction sites. Sequence mutations in INCENP (F825A and F837A) were generated by mutagenesis PCR using primer M4389 (F825A) and M4390 (F837A). Full-length Mad2 was amplified by PCR (M5700 and M5701) and subcloned into pEGFP-C2 through BamH1 and Sal1 restriction sites. To clone PICH constructs for expression in insect cells, full length PICH was amplified by PCR using pEGFP-C1-PICH WT (Baumann et al., 2007) as a template and primers M6343 and M6345 for full length PICH. An N-terminal His-tag was inserted and the construct was further cloned into the multiple cloning site I (MCS I) of the pFBDM vector through SalI and NotI restriction sites. For all primers used and a complete list of plasmid constructs that have been cloned during the course of this work see Appendix.

### 3. Bacmid preparation for protein expression in insect cells

Plasmid DNA of expression constructs was transformed by a heat shock at 42°C for 45 sec into chemical competent DH10MutliBac cells and incubated in a shaking incubator at 37°C for 4-6 h after the addition of 1 ml LB medium. Cells were plated on agar plates, containing the antibiotics kanamycin (50 µg/ml), gentamycin (10 µg/ml) and tetracycline (10 µg/ml), as well as X-α-galactose (100 µg/ml) and IPTG (40 µg/ml). Plates were incubated at 37°C for 48 h. Successful integration of the gene of interest into the bacmid was assessed by blue/white screening. White colonies were used to inoculate an overnight shaking culture of 150 ml LB medium containing respective antibiotics. Cells were harvested by centrifugation and the

bacmid isolated using the Midi-Prep Kit (Qiagen), following the instructions provided by the manufacturer.

**4. Virus generation, protein expression and purification in insect cells**

Freshly split Sf21 cells were transiently transfected with 2 µg of bacmid DNA using FuGene transfection reagent (Roche) according to the manufacturer's protocol. After incubation for 72 h at 27.5°C the supernatant (viral generation P0) was collected and viral titer was amplified by infecting 10 ml freshly resuspended Sf21 cells with 1 ml of P0. Cells were cultured in shaking flasks at 27.5°C and shaking at 85 rpm. After 72 h of incubation the supernatant was harvested by centrifugation (viral generation P1) and used for the final amplification of viral titer P2. 500 ml Sf21 cells were supplemented with 0.5 ml P1 virus and cultured as described previously.

For expression of N-terminally $His_6$-tagged PICH full length protein, H5 cells were harvested 72 h after baculovirus infection (10 ml P2 virus used for 500 ml H5 cells with a density of $1x10^6$ cells/ml), resuspended in lysis buffer (20 mM Hepes pH 7.5, 200 mM NaCl, 5% glycerol (v/v) and 5 mM β-mercaptoethanol) and lysed by sonifiaction. Cell debris was removed by centrifugation and a final concentration of 20 mM imidazole was added to the supernatant. His-tagged constructs were first purified by pre-equilibrated Ni-NTA agarose (Qiagen) packed in a gravity flow cartridge (BioRad). After loading of soluble extracts, Ni-NTA beads were washed with lysis buffer (wash 1), followed by a wash with lysis buffer supplemented with 30 mM imidazole (wash 2), and lysis buffer supplemented with 1 M NaCl (wash 3) before elution of the protein (lysis buffer supplemented with 200 mM imidazole). All fractions (except for washing step 3) were subsequently boiled in SDS sample buffer prior to SDS-PAGE and Coomassie Blue staining for gel analysis. The elution fraction was loaded on a pre-equilibrated 5 ml HiTrap Q Sepharose column (GE Healthcare). PICH was eluted by a linear gradient ranging from 200 mM NaCl (lysis buffer) to 1 M NaCl. Peak fractions containing PICH were pooled and loaded onto a Superdex 200 26/60 size exclusion chromatography column (GE Healthcare) in lysis buffer. Peak fractions containing PICH were pooled and concentrated with centrifugal devices (Amicon Ultra, Millipare) to the final concentrations of 2.6 µg/µl and 4.4 µg/µl.

**5. Live-cell imaging**

For imaging experiments, HeLa cells stably expressing histone H2B-GFP were used (Sillje et al., 2006). They were cultured in 8-well chamber slides (Ibidi) with 300 µl medium per well and transfected with siRNA oligonucleotides and/or plasmid constructs, as appropriate. 10 h post-transfection, they were synchronized using 2 mM thymidine for 24 h and then released into fresh medium for 8 h prior to imaging. Live-cell imaging was performed using a Zeiss Axiovert 2 microscope equipped with a Plan Neofluar 20x1.6 optivar/NA=0.75 objective, an environmental chamber and a CoolSNAP-ES2 digital camera system. Movies were acquired over at least 12 h with pictures taken at time intervals of 3 min. Images were captured with 2.5-5 ms exposure time for histone H2B-GFP, 30 ms for mCherry and 50 ms for DIC. Metaview software (Visitron Systems) was used for data collection and analysis.

**6. Western blotting**

Whole cell extracts were prepared using lysis buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 40 mM β-glycerophosphate, 10 mM NaF, 0.5% IGEPAL (v/v), 2 mM Pefabloc (Roth), 0.3 mM $NaVO_3$, 100 µM ATP, 100 µM $MgCl_2$, 100 nM okadaic acid, 1 protease inhibitor tablet per 10 ml lysis buffer (Roche)). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore) and these were probed with primary antibodies overnight at 4°C. Membranes were washed with 0.5% Tween/PBS before incubation with secondary antibodies. Antibodies used are listed in the Appendix.

**7. Immunofluorescence microscopy**

Cells were grown on coverslips, fixed with PTEMF (immunostaining for kinetochore associated proteins) for 10 min at room temperature (Stucke et al., 2002) or -20°C methanol (immunostaining for phosphor-specific antibodies) for 10 min at -20°C, and incubated for 20 min at room temperature in 3% BSA/PBS. Antibody incubations were carried out for 1 h at room temperature, followed by three washes in 0.5% TritonX-100/PBS. DNA was stained with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Primary antibodies for immunostaining are listed in the Appendix. Primary antibodies were detected by Cy3-conjugated donkey antibodies (Dianova) and Alexa Fluor 647-conjugated goat antibodies (Invitrogen). Coverslips were mounted in phenylenediamine in 90% glycerol (v/v). For image acquisition a Deltavision microscope (Applied Precision) on a Nikon Eclipse TE200 base (Applied Precision) equipped with an Apo 60/1.4 oil immersion objective and a CoolSnap HQ camera

(Photometrics) was used. Samples were examined with optical sections acquired 0.4 μm apart in the z-axis, deconvolved for each focal plane and projected into a single plane image using the Softworx software (Applied Precision). Images were processed in Adobe Photoshop CS3 and figures were assembled using Adobe Illustrator CS3 (Adobe Systems).

**8. Quantitative real-time PCR**

To monitor mRNA levels, HeLa cells were depleted of various proteins by siRNA and synchronized by a single thymidine block. To analyze the expression levels of Mad1, Mad2, PICH and Tao1 transcripts, total RNA was extracted using an RNeasy Mini kit according to manufacture's protocol (Qiagen) and quality controlled for integrity by capillary electrophoresis on Agilent 2100 Bioanalyser. Transcript levels were determined by qRT-PCR. First-strand cDNA synthesis was carried out from 1 μg of total RNA by using SuperScript II Reverse Transcriptase and random primers following the manufacturer's instructions (Invitrogen Life Technologies). The amplicons were designed with the program Primer Express 2.0 (Applied Biosystems, Foster City, CA) by using default parameters such that they spanned exon boundaries. Primer sequences are available upon request. Amplicon sequences where checked by BLAST against the human genome to ensure that they were specific for the gene being assayed. The specificity of each primer pair as well as the efficiency of the amplification step was tested by assaying serial dilutions of cDNA. PCR reactions were carried out in triplicate by using an SDS 7900 HT instrument (Applied Biosystems) and Power SYBR Green PCR master mix kits (Applied Biosystems). Normalization genes were selected using the geNorm script as published (Vandesompele et al., 2002). Raw Ct values obtained with SDS 2.0 (Applied Biosystems) were imported into Excel (Microsoft, Redmond, WA) to calculate the normalization factor and the fold changes with the geNorm (Vandesompele et al., 2002).

**9. Co-immunoprecipitation**

For co-immunoprecipitation of endogenous or overexpressed proteins, HeLa cells were released from a single thymidine block (unless otherwise stated) followed by mitotic shake-off. Cell pellets were washed in PBS and subsequently lysed in lysis buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 40 mM β-glycerophosphate, 10 mM NaF, 0.5% IGEPAL (v/v), 2 mM Pefabloc (Roth), 0.3 mM $NaVO_3$, 100 μM ATP, 100 μM $MgCl_2$, 100 nM okadaic acid, 1 protease inhibitor tablet per 10 ml lysis buffer (Roche) and 80 U/ml micrococcal nuclease

(Sigma)) for 30min on ice. Antibodies and corresponding IgG controls were coupled to Affi-Prep Protein G beads (Pierce Biotechnology) and incubated with cleared lysate for 2 h at 4°C on a rotating wheel. Immuno-complexes were washed 3 times in lysis buffer and subsequently boiled in SDS sample buffer prior to SDS-PAGE and Western blotting.

**10. *In vitro* kinase assay**

For *in vitro* kinase assays, the active Aurora B-INCENP complex (kind gift from Ulf R. Klein) and respective substrates at 100 ng were incubated in BRB80 buffer (80 mM Pipes pH 6.8, 1 mM MgCl2, 1 mM EGTA). Kinase reactions were carried out at 37°C for 45 min in these buffers supplemented with 10 μM ATP and 2 μCi [γ-32P] ATP (Amersham Corp.). Reactions were stopped by the addition of SDS sample buffer and heating at 95°C for 5 min. Protein samples were separated by SDS–PAGE followed by Coomassie Blue staining. The gels were dried on filter paper and $^{32}$P incorporation was visualized by autoradiography.

**11. Electrophoretic mobility gel shift assay (EMSA)**

Increasing amounts of purified recombinantly expressed PICH protein were incubated with 50 nM fluorescently labeled dsDNA (30mer oligonucleotide, forward sequence: 5'-6-FAM-CCGGAAAGCATCTAGCATCCTGTCAGCTGC), ssDNA (30mer oligonucleotide, poly dT) or DNA annealed to form a Holiday Junction structure (sequence 1: 5'-GGCGACGTGATCACCAGATGATGCTAGATGCTTTCCGAAGACAGACC, sequence 2: 5'-6-FAM-GCTCTCTCTTCGGAAAGCATCTAGCATCCTGTCAGCTGCATGGAACG, sequence 3: 5'-CGTTCCATGCAGCTGACAGGATGCTAGTCAAGGCGAACTGCTAACGG and sequence 4: 5'-CCGTTAGCAGTTCGCCTTGACTAGCATCATCTGGTGATCACGTCGCC). The reaction was done in EMSA buffer (20 mM Hepes pH 7.5, 150 mM KCl, 5% glycerol (v/v) and 5 mM β-mercaptoethanol) for 5 min on ice before addition of loading buffer (80% glycerol in Tris-HCl pH 7.5) and analysis on an 0.75% agarose gel. Detection of fluorescently labeled DNA was performed on a Typhoon 8600 variable mode imager (GE Healthcare).

**12. Inihibitors**

Aurora B kinase activity was efficiently blocked by using the inhibitor ZM447439 (Tocris Bioscience) at 10 μM or VX-680 (kindly provided by Patrick Eyers) at a concentration of 2 μM. SP600125 (BioMol) was applied with a final concentration of 10 μM to inhibit

Aurora B kinase activity. Plk1 kinase activity was inhibited by using 1 μM ZK-Thiazolidinone (TAL, Santamaria et al., 2007). Monastrol was used at a final concentration of 150 μM. The proteasome inhibitor MG132 (Calbiochem) was used at 10 μM and cells arrested in metaphase were obtained by treatment of 25 μM noscapine. Prometaphase arrest was achieved by treatment with 650 nM nocodazole or 200 nM taxol.

**13. Crystallization**

Protein crystallization trials were set up in commercial 96-well format sitting-drop screens (JBScreen Classic 1 and 2 (Jena Bioscience), NeXtal Classics Suite and NeXtal ProComplex Suite (Qiagen)) at 16°C with protein concentration of 2.6 μg/μl and 4.4 μg/μl. The crystal phoenix robot (Art Robbins Instruments) was used to dispense both the reservoir and the drops. The reservoir contained 50 μl screening solution, and drops were mixed from equal volumes of reservoir solution and protein (final drop volume 400 nl). The 96-well plates were analyzed for crystals every 3-5 days over two months.

# VII. APPENDIX

### 1. List of siRNA oligonucleotide sequences

All siRNA oligonucleotides sequences were ordered from Qiagen if not stated otherwise.

Aurora B: (Klein et al., 2006)

Blinkin: (Kiyomitsu et al., 2007)

Borealin: (Klein et al., 2006)

Bub1: 5'-CCAGTGAGTTCCTATCCAATT

BubR1: 5'-AAGTCTCACAGATTGCTGCCT

CENP-E: 5´-ACTGGAGAGCAGTAAGAGTTT

Eg5: (Baumann et al., 2007)

GL2: (Elbashir et al., 2001)

Hec1: 5'-AAGTTCAAAAGCTGGATGATC

INCENP: (Klein et al., 2006)

Mad1: (Stucke et al., 2002)

Mad2: (Baumann et al., 2007)

Mps1: (Stucke et al., 2002)

Nuf2: (DeLuca et al., 2002)

PICH-1: (Baumann et al., 2007)

PICH-2: (Baumann et al., 2007)

PICH-CC: (Leng et al., 2008)

PICH-3: 5'-AGUAGGUGGUGUCGGUUUA (Dharmacon ON-TARGETplus)

PICH-4: 5'-GGAUAGAGUUUACCGAAUU (Dharmacon ON-TARGETplus)

PICH-5: 5'-CCAGAAACCUCAAUCGGAU (Dharmacon ON-TARGETplus)

PICH-6: 5'-ACUUUAAGACAUUGCGAAU (Dharmacon ON-TARGETplus)

PICH SMART pool (PICH-SP) contained a mixture of PICH-3, -4, -5 and -6 siRNAs

Plk1: (Baumann et al., 2007)

Sgo1: 5'-GAACACATTTCTTCGCCTATT

Sgo2: 5'-TCGGAAGTGTAATTTCTTATT

Ska1: (Hanisch et al., 2006a)

Tao1-NCB3: (Draviam et al., 2007)

Tao1-2: 5'-CCAAGUAUCUCGUCACAAA (Dharmacon ON-TARGETplus)

Tao1-3: 5'-GUAAUAUGGUCCUUUCUAA (Dharmacon ON-TARGETplus)

Tao1-4: 5'-CUAAAGUGAUGUCCAAUGA (Dharmacon ON-TARGETplus)

Tao1-5: 5'-GCUGUGAGUUGAUCAGAUU (Dharmacon ON-TARGETplus)

Tao1 SMART pool (Tao1-SP) contained a mixture of Tao1-2, -3, -4 and -5 siRNAs

**2. List of primers**

M3863-TCTCCCCGGGATGGAGGCATCCCGAAGGTTTC

M3864-ATAAGAATGCGGCCGCTCAATTGTTATTAAGTTGC

M3821-GATGATATGGGATTAGGGGCGACTGTTCAAATCATTGCT

M3822-AGCAATGATTTGAACAGTCAGTCGCCCCTAATCCCATATCATC

M3914-CGGGGTACCGCCACCATGGTGAGCAAGGGCGAGGAGGAT

M3915-GCGATATCCTTGTACAGCTCGTCCATGCCG

M3344-GAGGGTGAGAAACAAGACTTATCCAGTATAAAGGTG

M3345-CACCTTTATACTGGATAAGTCTTGTTTCTCACCCTC

M3666-GATCCC*CAAGATCTCTCCAGTATA*TTCAAGAGA*TTATACTGGAGAGATCT TG*TTTTTGGAAA

M3667-AGCTTTTCCAAAAA*CAAGATCTCTCCAGTATAA*TCTCTTGAA*TTATACTGG AGAGATCTTG*GG

(Italic letters indicate the siRNA sequence published for siPICH-1.)

M5700-CGCGGATCCAGATGGCGCTGCAGCTCTCCCGGGAGCAGGG

M5701-CGCGTCGACTCAGTCATTGACAGGAATTTTGTAGGCC

M4389-CGAACCTTCTGGAGCTCGCCGGAACCATTCTCCCAC

M4390-GACTTGGAGGATATCGCCAAGAAGAGCAAGCCC

M6343-AAAAAGTCGACATGAAACATCATCATCATCATCATAAACTCGTGCCAAGA GGCTCTATGGAGGCATCCCGAAGGTTTCCGG

M6345-AAAAAGCGGCCGCTCAATTGTTATTAAGTTGC

## 3. List of plasmid constructs

### 3.1. Expression in insect cells

| vector | DNA insert (MCS I) | DNA insert (MCS II) | tag | P2 virus | expression in H5 |
|---|---|---|---|---|---|
| pFBDM | PICH 1-1250 | - | N-term His | yes | soluble |
| pFBDM | PICH 1-752 | - | N-term His | no | n/a |
| pFBDM | PICH 1-715 | - | N-term His | yes | insoluble |
| pFBDM | PICH 1-632 | - | N-term His | no | n/a |
| pFBDM | PICH 1-571 | - | N-term His | not tested | n/a |
| pFBDM | PICH 1-454 | - | N-term His | yes | insoluble |
| pFBDM | PICH 1-421 | - | N-term His | not tested | n/a |
| pFBDM | PICH 79-1250 | - | N-term His | yes | soluble |
| pFBDM | PICH 79-752 | - | N-term His | no | n/a |
| pFBDM | PICH 79-715 | - | N-term His | yes | soluble |
| pFBDM | PICH 79-632 | - | N-term His | not tested | n/a |
| pFBDM | PICH 79-571 | - | N-term His | no | n/a |
| pFBDM | PICH 79-454 | - | N-term His | yes | insoluble |
| pFBDM | PICH 79-421 | - | N-term His | yes | insoluble |
| pFBDM | PICH 1-1250 | Plk1 | N-term His | yes | no PICH |
| pFBDM | PICH 79-1250 | Plk1 | N-term His | yes | no PICH |

Only pFBDM-PICH 1-1250 has been used for crystallization and EMSAs during the course of this work. Protein purification of shorter constructs was not possible due to low protein expression.

### 3.2. Expression in mammalian cells

| vector | DNA insert | provided by | tag |
|---|---|---|---|
| pEGFP | Mad2 | | GFP |
| pcDNA | INCENP$^{F825A/F837A}$ | | myc |
| pcDNA | INCENP$^{1-50}$ | Ulf R. Klein | myc |
| pcDNA | INCENP$^{wt}$ | Ulf R. Klein | myc |
| pTER+ | shPICH-1 | Lily H.-C. Wang | myc |
| pcDNA4/TO | PICH WT | Lily H.-C. Wang | mCherry |
| pcDNA4/TO | PICH-K128A | Lily H.-C. Wang | mCherry |
| pcDNA4/TO | PICH-T1063A | Lily H.-C. Wang | mCherry |

## 4. List of primary antibodies

### 4.1. Immunofluorescence

mouse anti-Aurora B (AIM1, BD Transduction)

mouse anti-Blinkin (Kiyomitsu et al., 2007)

mouse anti-Bub1 (in-house production)

mouse anti-BubR1 (in-house production)

rabbit anti-Ser7-phospho-CENP-A (Upstate)

CREST (Immunovision)

mouse anti-Cyclin B1 (in-house production)

mouse anti-Hec1 (GeneTex)

mouse anti-INCENP (in-house production)

mouse anti-Mad1 (Sigma)

rabbit anti-Mad2 (Bethyl)

mouse anti-myc (9E10, in-house production)

rat anti-PICH (Baumann et al., 2007)

mouse anti-Plk1 (in-house production)

rabbit anti-Plk1 (Abcam)

mouase anti-Sgo1 (Abnova)

rabbit anti-Sgo2 (Bethyl)

mouse anti-Ska1 (in-house production)

mouse anti-γ-tubulin (Sigma)

### 4.2. Western blotting

mouse anti-Aurora B (AIM1, BD Transduction)

mouse anti-Blinkin (Kiyomitsu et al., 2007)

rabbit anti-Borealin (Klein et al., 2006)

mouse anti-Bub1 (in-house production)

mouse anti-Bub3 (BD Transduction)

mouse anti-BubR1 (in-house production)

mouse anti-Cdc20 (Chemicon)

mouse anti-Cdc27 (BD Transduction)

mouse anti-Cyclin B1 (Upstate)

mouse anti-GFP (in-house production)

mouse anti-Hec1 (GeneTex)
histone H3-phospho-serine 10 (Upstate)
mouse anti-Mad1 (Sigma)
rabbit anti-Mad2 (Bethyl)
monoclonal anti-Mps1 (Stucke et al., 2002)
mouse anti-myc (9E10, in-house production)
rabbit anti-PICH (Baumann et al., 2007)
mouse anti-Plk1 (in-house production)
rabbit anti-Survivin (Biozol)
rabbit anti-Tao1 (Bethyl)
monoclonal anti-α-tubulin (DM1A, Sigma)
rabbit-IgG (Invitrogen)
mouse-IgG (Invitrogen)

We thank Andreas Uldschmidt, Elisabeth Bürgelt and Alicja Baskaya for the in-house antibody production.

**5. Abbreviations**

All units are abbreviated according to the International Unit System.

APC/C: anaphase-promoting complex/cyclosome
ATP: adenosine-triphosphate
A.U.: arbitrary units
BSA: bovine serum albumin
Blinkin: Bub-linking kinetochore protein
Bub: budding inhinhibited by benzimidazole
Cdc: cell division cycle
Cdk: cyclin-dependent kinase
CENP: centromeric protein
CIP: calf intestine phosphatase
CPC: chromosomal passenger complex
C-terminus: carboxy-terminus
DAPI: 4´,6-diamidino-2-phenylindole

DIC: differential interference contrast microscopy

DMSO: dimethyl sulfoxide

dsDNA: double stranded deoxyribonucleic acid

EMSA: electrophoretic mobility shift assay

FACS: fluorescent activated cell sorting

FBS: fetal bovine serum

GFP: green fluorescent protein

GST: glutathione S-transferase

h: hour(s)

Hec1: highly expressed in cancer protein 1

H2B: histone H2B

HJ: Holiday Junction

IAP: inhibitor of apaptosis

IgG: immunoglobulin G

INCENP: inner centromere protein

INO80: inositol requiring 80

IPTG: isopropyl-beta-D-thiogalactopyranoside

kDa: kilodalton

KT: kinetochore

Mad: mitotic arrest deficient

MAP: mitogen-activated protein

MARKK: microtubule affinity-regulation kinase kinase

MBP: maltose binding protein

MCAK: mitotic centromere-associated kinesin

MCS: multiple cloning site

min: minute(s)

Mps1: multipolar spindle-1

mRNA: messenger ribonucleic acid

MT: microtubule

NEBD: nuclear envelope breakdown

N-terminus: amino-terminus

PBD: polo-box binding domain

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFD: PICH family domain

PICH: Plk1-interacting checkpoint helicase

Plk: polo-like kinase

PP1: protein phosphatase 1

qRT-PCR: quantitative real-time polymerase chain reaction

RNAi: RNA interference

rpm: rounds per minute

SAC: spindle assembly checkpoint

SDS-PAGE: sodium dodecylsulfate polyacrylamid gelelectrophoresis

SF: superfamily

shRNA: short hairpin ribonucleic acid

siRNA: small interference ribonucleic acid

SNF: sucrose non-fermeting

ssDNA: single stranded deoxyribonucleic acid

SWI: mating-type switching

TAL: ZK-Thiazolidinone

Tao1: Thousand and one amino acid protein 1, also known as MARKK

TPR: tetratrichopeptide repeats

wt: wildtype

# VIII. REFERENCES

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| 12/2007-03/2010 | **PhD Scholarship**<br>From the Boehringer Ingelheim Fonds |
| 04/2006-08/2006 | **Scholarship**<br>From the German academic exchange Service (Deutschen Akademischen Austauschdienst) for studying abroad |
| 08/2004-07/2005 | **Scholarship**<br>From the Erasmus exchange Program |

## Publications

Hübner, N.C., Wang, L.H.-C., Kaulich, M., Descombes, P., Poser, I., and Nigg, E.A. (2010) Re-examination of siRNA specificity questions role of PICH and Tao1 in the spindle checkpoint and identifies Mad2 as a sensitive target for small RNAs. Chromosoma 119, 149-165.

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